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CULTIVATION AND ISOLATION OF GONOCOCCI *

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This work was undertaken to find, if possible, a simple medium which would give uniform results and at the same time prolong the life of the gonococcus in culture. We used 15 strains of gonococci from recent cases of gonorrhea.

Numerous mediums were tried for isolating gonococci. The medium used most is made as follows: Beef or veal infusion-agar is prepared containing 1.6% agar; adjusted to a P_H of 7.6. This is divided in 100 c c quantities in Erlenmeyer flasks and sterilized. These flasks are kept as stock medium for making the plates. A flask containing the infusion-agar is heated until the agar is melted; while it is still hot (90-100 C.) 30 c c of beef serum is added and thoroughly mixed so that the serum, coagulated in small particles, is distributed evenly through the medium; this is then poured into petri dishes, covered with porous terra-cotta lids and placed in the incubator over night (about 18 hours); the terra-cotta lids are replaced by glass lids at the end of this time, and the plates are ready for inoculation.

The type of serum used seems to have little influence—human, rabbit, sheep, and calf serum all giving about the same results. On plates that were quite moist we generally found a good growth but it is short lived. On the other hand there was a degree of dryness which appeared to inhibit growth; if the terra-cotta lids were left on for a period longer than 24 hours, the results were not satisfactory, and if the drying with the porous lids was continued as long as 72 hours, before the inoculation was made, the growth was uncertain, scanty and short lived. Varying proportions of serum were used, but it was found that 30 c c of serum to 100 c c of the infusion-agar gave the best results. In isolating the culture of gonococcus the discharge was taken from the patient directly on a sterile platinum loop and streaked thinly over the surface of the plates. The plates were warmed to body temperature before the inoculation was made and were immediately transferred to the incubator. The colonies of gonococcus appeared

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in from 18 to 30 hours, reaching their maximum growth in about 48 hours. The first growth often began to die out in 72 hours and transplants after that time often proved unsuccessful. Probably the initial growth does not survive beyond four days; this is due, at least in part, to overgrowth by other organisms—in particular a small gram-negative bacillus. The organism can usually be transferred from the first plate to a medium which does not contain serum; but a serum transplant is usually advisable, as a few strains grow very poorly at first in the absence of serum.

In studying the cultivation of the gonococcus, we tried a number of mediums previously recommended. We concerned ourselves principally with the duration of life of the organisms in artificial mediums, believing that a consideration of this factor would give us valuable information regarding the needs of the organism.

1. Plain beef infusion-agar adjusted to a P_H of 7.6 was first used. Results after 48 hours of incubation showed a scanty growth in some and no growth at all in the majority of the tubes. Freshly isolated cultures gave no growth or scanty growth when transplanted into this medium. This medium is evidently a poor one even for older cultures.

2. Plain beef extract agar gave no growths or very poor growth.

3. Plain liver infusion-agar gives better growths than either beef infusion or beef extract agar. Growth is, however, short lived.

4. Thallmann's agar¹ is beef infusion agar prepared in the usual way, except that it is boiled for 45 minutes over a bath of saturated sodium chloride solution. In practice we have found that Thallmann's method of adjusting the reaction gives a P_H concentration of 7.3-7.5 and the regular adjustment of the reaction of the medium to this P_H concentration proved very satisfactory.

It was found that for certainty and abundance of growth this is perhaps the best of all the solid mediums we have used. Slants of this medium dried so that the water of condensation has almost disappeared give regularly abundant growths with all the strains we have isolated. The duration of the life of this growth is not great under the most favorable circumstances, usually being 3 to 4 days, occasionally extending to 10 days, so that it is not satisfactory for the preservation of stock cultures.

To ascertain the proper degree of moisture needed for the cultivation of the gonococcus in this medium, we transplanted the organisms

¹ Centralbl. f. Bakteriöl., I. O., 1900, 27, p. 828.

into tubes which had been in the incubator from 1 to 10 days before inoculation. It was found that the tubes that retained a small amount of water of condensation at the time of inoculation showed the most profuse growth, and that the tubes in which the medium was hard and even cracked showing scanty growths or no growths at all. The tubes in which the cultures survived longest were also those in which the water of condensation had not quite disappeared at the time of inoculation.

5. Vedder's starch medium² is adjusted to a P_H concentration of 7.5 and consists essentially of the infusion-agar to which 1% corn starch is added. On this medium the growth is of almost equal abundance to that on Thallmann's agar; the growth lives longer, however, in this medium than in Thallmann's. This was especially noted when the tubes were sealed after 48 hours' incubation.

6. Dorsett's egg medium³ has not given satisfactory growths in the strains of gonococcus we have isolated. The addition of 5% glycerol to this medium failed to improve the growth.

7. Meat infusion gelatin gave unsatisfactory results. At 20 C. no growths were obtained, and it was exceptional to obtain a growth at 37.5 C.

8. Huntoon's hormone medium⁴ gives fairly satisfactory growths but scarcely as abundant as Vedder's starch medium or Thallmann's agar.

9. North's gelatin-agar medium with nutrose⁵ gives excellent growths, but has not proved superior to Vedder's starch medium or to Thallmann's agar.

10. Whole blood agar prepared by adding defibrinated rabbit or human blood to agar either at 55 C. or at higher temperatures does not appear to have any advantage over serum mediums.

Although some of the solid mediums gave satisfactory growths with all our strains, not one of them was found useful for the lengthy preservation of stock cultures; the growth although abundant was short-lived, surviving from 4 to 10 days without transplantation. We turned our attention then to fluid mediums to find one satisfactory for the preservation of stock cultures. The following liquid mediums were

² Jour. Infect. Dis., 1915, 16, p. 385.

³ Am. Med., 1902, 3, p. 555.

⁴ Jour. Infect. Dis., 1918, 23, p. 169.

⁵ Jour. Med. Res., 1909, 20, p. 359.

tried, a P_H concentration of 7.3 being used in all these experiments, as it appeared to be the optimum concentration in those types in which growth occurred.

1. Plain meat infusion broth, made in the usual way, rarely gave any growth; the addition of 2% glucose did not improve the mediums.

2. Plain infusion broth with the addition of various amounts of serum up to 30% did not give satisfactory growths with any regularity, nor did the addition of the serum at from 90 to 100 C. improve the growth.

3. Meat infusion broth boiled for 45 minutes over saturated sodium chloride solution also gave poor results in our hands. The addition of 1% nutrose, or 1 to 6% gelatin, did not make this a better medium.

4. Meat infusion broth, boiled over saturated salt solution for 45 minutes, with the addition of agar 0.5% gave good growths, the organisms living from about 7 to 14 days. Only 3 cultures of 14 did not survive for 8 days or more and 2 survived 14 days.

5. Meat infusion broth, boiled over saturated salt solution for 45 minutes, with the addition of agar, 0.5%, and nutrose, 1%, gave essentially the same results as the medium just described, with the exception that the growth was more abundant.

6. The results obtained in the 2 mediums last described led us to try variations of this type of medium, and as a result we developed the following medium which has proved satisfactory: 500 gm. finely chopped beef, and 1,000 c c of distilled water are mixed and set in the icebox for 24 hours, and then strained through a linen towel. To 1,000 c c of infusion add peptone, 1%, and salt, 0.5%. Boil to clear; filter through filter paper. Now add agar, 0.5%, gelatine, 5%, and nutrose, 1%. Boil over saturated sodium chloride solution in a double boiler for 45 minutes. Strain through absorbent cotton; titrate to P_H 7.3. Tube and sterilize in the autoclave for 20 minutes. This gives a semisolid medium.

The growth in this medium begins as a thin film on the surface radiating from the site of inoculation; it extends slowly, finally covering the entire surface and forming an opaque layer with fine threadlike extensions into the deeper portions.

Of 14 strains grown on this medium 6 survived between 30 and 32 days and 8 survived 20 or more days, 4 of the latter beyond the 25 days. We found that sealing the tubes aided in the survival of the

gonococci in the medium. Growth also appeared to be better when the organisms were deposited on the surface of the medium and when the tubes were sealed after 48 hours' incubation.

We substituted 1% corn starch for the nutrose in the preparation of the semisolid mediums, and also 1% potato starch, but we did not find it to be any better than nutrose. Although growth occurs, it is difficult to distinguish it on account of the cloudiness of the medium. Cultures in these mediums are not as long lived, possibly because the medium tends to dry and cake at the surface.

Since in spite of all variations of moisture, P_H concentration and composition of the solid mediums that we have been able to try, the most vigorous strains have not survived longer than 2 weeks without transplanting, we regard this semisolid medium as the most satisfactory as far as our experiments have been developed for the preservation of stock cultures of gonococci. The following facts must be noted: Subcultures should be made from the surface growth. The tubes should be kept in the incubator at 37.5 C. during the entire time; keeping the tubes in the icebox or at room temperature does not permit the organism to survive as long. It has been found advantageous to seal the stock culture tubes with paraffin after growth has progressed for 24 hours. This prevents too rapid drying during the period of incubation.

Experiments with the various mediums which we have used has convinced us that the optimum hydrogen-ion concentration for the growth of the gonococcus in culture mediums lies between P_H 7.3 and 7.6. We prefer P_H 7.3 for liquid mediums and use 7.6 for solid mediums. The gonococcus can, however, become accustomed to growth through a fairly wide range of P_H concentration. We have successfully grown it from P_H 7 to 8.2. Sudden variations, as a change from P_H 7.3 to 8.1, are usually not well borne, especially by certain strains. But the change can be made gradually in practically all of our strains.

DISCUSSION

Our object was to test the value of various culture mediums for the isolation and maintenance in stock of cultures of gonococci. Possibly we must differentiate between organisms immediately isolated from the human host, and organisms which have grown in cultures for even one generation—it may be that the former are more fastidious, but this is by no means certain. It is certain, of course, that the longer the gonococci exist on artificial medium, the better they become accustomed

to the conditions, and this is a factor which must be taken in consideration in studying these and probably other similar organisms. We cannot, therefore, directly draw conclusions regarding isolation of gonococci from experiments with older stock cultures. In the main, our experiments were carried out while the strains of gonococci were still relatively recently isolated. It was evident while certain simple mediums, such as Thallmann's medium and Vedder's starch medium, were suitable for the maintenance of stock cultures, that other simpler mediums such as ordinary nutrient broth or nutrient agar were not suitable. The fact that the gonococci grew quite well on the hormone mediums makes it quite evident that the gonococci do not require complex chemical substances, such as the true proteins, for their growth.

In isolating the gonococcus, however, we did find it advisable to add serum to our mediums. Whether serum might be replaced by some other substance of simpler chemical nature, remains to be determined. Given, then, a medium of a sufficient nutrient value, it appears that physical factors play a most important part in the successful cultivation of gonococci.

Temperature is undoubtedly of importance—a temperature of 42 C. has destroyed our cultures which were exposed from 16 to 20 hours. In the early periods after isolation cultures of gonococci should not be incubated at a temperature above 39 C. Temperature has also influence on the longevity of stock cultures, and we found it best to keep the stock cultures when testing duration of life in the incubator rather than at room temperature or in the icebox.

There is probably an optimum degree of moisture of the growth of gonococci, as suggested by Warden.⁶ We found that the gonococci did not grow nor survive long in moist or fluid mediums, and we noted the most profuse growth and longest survival on the semisolid mediums. On the other hand, on dry mediums the gonococcus grew poorly if at all. We made some experiments in which we left the cultures in jars with water and compared the growth here with the growth in the moist incubator, but we were unable to note any distinct beneficial effect of cultivation in the moist jar.

The influence of the hydrogen-ion concentration of the medium is well recognized generally.

From our results with semisolid mediums, which gave such excellent effect in prolonging the vitality of the organisms, we incline strongly

⁶ Jour. Infect. Dis., 1913, 12, p. 93; 13, p. 124; 1915, 16, p. 426; Jour. Am. Med. Assn., 1917, 68, p. 432.

to the idea that we are dealing here principally with a physical effect. Thallmann's agar, similar in composition but different in physical qualities, did not serve to prolong the life of the gonococcus as did the agar-gelatin-nutrose mediums. Unquestionably the prolongation of life bespeaks a more favorable medium. What the factor or factors are which are here concerned is not certain. Whether we deal in this medium with influences on the cell membrane, such as Warden⁶ suggests in his studies, or whether we are concerned with factors similar to those affecting the value of Hitchens'⁷ 0.1% agar medium, namely, colloidal dispersion, or whether we deal with viscosity, surface tension, protective colloid reactions, diffusion or osmotic phenomena, cannot be stated. Possibly physical factors are of as much, if not more, importance in the cultivation of gonococcus than are nutritional factors; it may be that this statement may hold true as well of other organisms.

SUMMARY

For isolation of the gonococcus we found the use of a 1.6% agar of P_H 7.6 to which 30% beef serum was added while the agar was still hot (90-100 C.), quite satisfactory. It is important that the plates are not too moist when used.

A study of various culture mediums has shown that Thallmann's agar medium and Vedder's starch medium are excellent for the cultivation of gonococcus.

On semisolid 0.5% agar, 5% gelatine, 1% nutrose, the gonococcus has grown and survived for from 3 to 4 weeks.

It appears that physical factors are possibly of equal importance with nutritional factors in the cultivation of the gonococcus.

⁷ Jour. Infect. Dis., 1921, 29, p. 390.

IMPROVED METHODS FOR THE ISOLATION AND LATER CULTIVATION OF *B. PERTUSSIS*

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The need of more strains of *B. pertussis* for inquiry as to the presence of types and the possibility of making a more effective vaccine was the starting point of this study. It was difficult to obtain material in the beginning because there were not many cases of whooping cough in New York City during the winter of 1921-1922. Those reported were usually too advanced for the isolation of the organism. We were, therefore, on the lookout for secondary cases in the families in which pertussis had been reported, but in most instances we were able to obtain sporadic cases only.

Bordet and Gengou emphasize the fact, corroborated since by many, that it is a comparatively easy task to isolate *B. pertussis* in the early stages of the disease. In the first ten days under favorable conditions, an almost pure culture of *B. pertussis* is frequently obtainable.

In general, the favorable factors include: (1) a sputum which has been obtained at the end of the characteristic spasmodic cough; (2) the washing of the sputum a few times in salt solution; after which a suspension for plating is made of any characteristic balls present; (3) freshly prepared plates of Bordet-Gengou medium made with defibrinated or citrated blood in the proportion of 1:3. The blood must be added at a temperature of from 45 to 50 C. and well mixed with the agar so that the plates will appear smooth, uniform and of a proper red color.

It is hard to get sputum from young children unless it is expelled in a fit of spasmodic coughing, or is removed from the mouth or throat with a spatula by depressing the tongue before the child has had a chance to swallow after the coughing spell.

The medium in use in this laboratory for isolation of *B. pertussis* is that of Bordet-Gengou¹ modified slightly in a few points: thus 2% instead of 1% glycerol is added; horse defibrinated or citrated blood instead of human or rabbit blood is used. The original formula of

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¹ Ann. d. l'Inst. Pasteur, 1906, 20, p. 731.

Bordet-Gengou does not give the reaction of the medium. Looking over the literature on the isolation of *B. pertussis*, we find that Arnheim² is the only one who mentions the reaction which he says is feebly acid, but he does not say which indicator he used to ascertain this point. The unadjusted Bordet-Gengou medium is feebly acid to litmus, but when tested as to the H-ion concentration, it is found to be P_H 5.8 to 6.1, that is, rather acid. In our laboratory the medium was originally adjusted by adding 8 c.c. of normal sodium hydroxid to each liter; this made the reaction about neutral to phenolphthalein before sterilization and supposedly slightly acid after sterilization. Williams, Wilson and Mishulow adjusted their Bordet-Gengou medium to this reaction for the isolation of *B. pertussis* in 1912 (personal communication). Recent tests of such a medium have shown that it may vary in reaction from P_H 7 to 7.8; usually it is about 7.4 to 7.6. In the beginning of the present work, we used a Bordet-Gengou medium adjusted to 7.2 to 7.4.

TECHNIC

From 6 to 8 plates of Bordet-Gengou medium and 3 "chocolate" plates made with Avery's³ sodium oleate agar were used for each case. The latter were useful for the purpose of determining the percentage of *B. influenzae* present. The Bordet-Gengou plates were incubated for from 48 to 72 hours and occasionally for 4 days. The colonies picked for fishing into Bordet-Gengou tubes were the minute whitish colonies, preferably those which showed a lightened area of the surrounding medium. In 48 hours, smears were made from all pertussis-like cultures (usually a whitish nonspreading growth), stained by gram and examined under the microscope. If the smears showed small ovoid, uniform gram-negative rods, the culture was transplanted to both chocolate and plain agar. If no growth occurred on either of these agars and if at the same time a good growth occurred on the Bordet-Gengou medium, the growths were considered culturally *B. pertussis*. They were then turned over to Miss Mishulow⁴ for further identification and typing by known pertussis serums.

In most cases from which *B. pertussis* was isolated the culture was obtained pure at the first attempt, but occasionally it was necessary to replate it a second or third time.

For these cases Bordet-Gengou medium (reaction P_H 7.2 to 7.4) was used exclusively. In this series we succeeded in obtaining 8 isolations of *B. pertussis*. The period of the disease in which the successful isolations occurred varied from the first to the third week of the whoop: In the first week, 3 isolations of 7 cases; in the second, 4 of 14 cases; in the third, 1 of 7 cases.

² Berl. klin. Wchnschr., 1908, 45, p. 1453.

³ Jour. Am. Med. Assn., 1918, 71, p. 2050.

⁴ Krumwiede, Mishulow and Oldenbush, Jour. Infect. Dis., 1923, 32, p. 22.

The cases were usually in the postcatarrhal stage. The statements of the patient's family were not always reliable, for the parents were not clear as to the distinction between the beginning of the cough and the beginning of the whoop.

This series comprised uncomplicated whooping cough only. Cases complicated with pneumonia, bronchopneumonia and exantheams are not included. Neither are any suspicious cases in which the patient devel-

FIRST SERIES; 35 CASES (TABLE 1)

TABLE 1
ISOLATION OF B. PERTUSSIS ON BORDET-GENGOU MEDIUM, REACTION PH 7.2-7.4

First Series of Thirty-Five Cases		
Number of Cases	Week of Whoop	Number of Isolations
7.....	1	3
14.....	2	4
7.....	3	1
7.....	4	0

TABLE 2
TABULATION OF ISOLATIONS OF B. PERTUSSIS REPORTED BY VARIOUS AUTHORS

Author and Year	Number of Whooping-Cough Cases	Stage	Isolations	
			Number	Percentage
Arnheim, 1908.....	20	Not mentioned	6	30
Fraenkel, 1908.....	38	Not mentioned	8	21
Seiffert, 1909.....	16	Early	12	75
Wollstein, 1909.....	20	From catarrhal to seventh week	5	25
Williams, 1912.....	187	From first to fourth week	27	14½
Poleff, 1913.....	14	Not mentioned	3	14½
Odaira, 1911.....	42	Not mentioned	5	12
Inaba, 1912.....	78	Early	68	88
Menschikoff, 1909.....	94	From beginning till eighth week	93	Nearly 100
Klimenko, 1908.....	76	5	6½
Chievitz and Meyer, 1916 (first series)....	126	From catarrhal to fifth and sixth weeks	67	53
Chievitz and Meyer, 1916 (second series)....	165		69	41.8
Shiga, 1913.....	Not given	Not mentioned	10	
Giese, 1918.....	19	Not mentioned	5	26.3

oped a cough after a course of prophylactic treatment with pertussis vaccines. These children did not prove to have pertussis eventually. In neither of these groups was B. pertussis isolated.

The percentage of successful results obtained with our first series, namely, 8 isolations from 35 cases (over 22.8%) correspond more or less with the results of most other workers, with a few exceptions (see table 2). In contrast to the majority of workers, Menschikoff⁵

⁵ Russki Wratch, 1909, 31, p. 1044.

reported almost 100% of isolations up to the eighth week of the disease. Inaba⁶ from 88 to 91%; Seiffert,⁷ early cases, 75%; Chievitz and Meyer,⁸ from 53 to 41%. The first two workers do not state whether they verified their cultures by serologic tests for complete identification.

Menschikoff used Bordet-Gengou medium to which hemoglobin in the proportion of 1:3 was added. The hemoglobin was obtained by freezing and thawing the blood and keeping it at a temperature above 0 C. for 24 hours.

Inaba's medium was a plain 3% agar solution to which defibrinated goat blood was added, 1:4. He does not mention whether he used salt or water with the agar and omits reference to the reaction.

Shiga⁹ isolated 10 cultures of *B. pertussis*; he does not state from how many cases. His medium was a modified Bordet-Gengou, that is, 1% peptone was added and the reaction made slightly alkaline (to what?). After the blood was added, the tubes or plates were heated at 56 C. for one half hour. This medium is so favorable for the isolation of *B. influenzae* that it is hard to understand how Shiga obtained pure cultures of *B. pertussis*. Perhaps this is the reason why this worker thinks that the two organisms are closely related and belong to one group.

Arnheim isolated 6 pertussis cultures from 20 cases and Frankel¹⁰ 8 from 38 cases. Frankel also reports that he isolated *B. pertussis* from 2 cases which were not pertussis clinically. Wollstein¹¹ had 5 isolations from 20 cases. Williams, Wilson and Mishulow had 27 isolations from 187 cases (14.5%) [unpublished], Poleff¹² 3 isolations from 14 cases, Odaira¹³ 5 isolations from 42 cases, Klimenko¹⁴ 5 isolations from 76 cases, and Giese¹⁵ 5 isolations from 19 cases.

An analysis of the 8 isolations in our first series, brings out some facts which are interesting, for they illustrate the importance of the stage of the disease in attempts to isolate the bacillus.

The first culture of *B. pertussis* was isolated from a child of 18 months. A sister of 5 years and a brother of 6 were reported to us as

⁶ Ztschr. f. Kinderheilk., 1912, 4, O., p. 252.

⁷ München. med. Wchnschr., 1909, 56, p. 131.

⁸ Ann. d. l'Inst. Pasteur, 1916, 30, p. 503.

⁹ Centralbl. f. Bakteriöl., I. O., 1913, 69, p. 104.

¹⁰ München. med. Wchnschr., 1908, 55, p. 1683.

¹¹ Jour. Exper. Med., 1909, 11, p. 43.

¹² Centralbl. f. Bakteriöl., I. O., 1913, 69, p. 23.

¹³ Centralbl. f. Bakteriöl., I. O., 1911, 61, p. 289.

¹⁴ Russki Wratch, 1908, 19, p. 637.

¹⁵ Ann. d. l'Inst. Pasteur, 1918, 32, p. 521.

having whooping cough. These 2 children had been whooping for over 2 weeks and the results with the sputum were negative. On a visit to the home of this family, it was discovered that the little sister of 18 months had just started to whoop. There was no difficulty in isolating the organism from the sputum. Of 5 cases in a school for colored children, *B. pertussis* was obtained in the case of one child only—a boy of 7. On a visit to the family of one of these children, a child of 2 was found to be coughing and had just begun to whoop. This case had not been reported to us. The sputum of this child gave us our third *B. pertussis* culture.

The fourth case which gave positive results was reported as suspicious whooping cough chiefly because the sister had the disease in a late stage. In the case reported, that of a girl of 10, there was a dry cough. No culture was obtainable by the droplet method recommended by Chievitz and Meyer. This method consists of making the patient cough on a Bordet-Gengou plate held a few inches from the face. In a few days the dry cough loosened up and an almost pure culture of *B. pertussis* was obtained from the sputum seeded on Bordet-Gengou medium.

The fifth culture was isolated from a boy of 3 years. No sputum could be obtained in the first attempt. Three days later sputum mixed with fresh vomit was brought to us; from this material we succeeded in isolating *B. pertussis*. A second culture taken one week later from this patient proved to be negative. The sixth and seventh cultures of *B. pertussis* were obtained from children in the Bellevue Clinic by the courtesy of Dr. Chaplin. One child was in the first stage of the disease, the second in the third week. The sister and brother of the first child who were in more advanced stages of the disease gave negative results. The eighth and last culture of this series was obtained from 1 of the 4 children examined in a home for children where there was an epidemic of whooping cough.

The greatest obstacle in isolation of *B. pertussis* was the accompanying growth of *B. influenzae*. The latter organism was isolated from almost every case of pertussis; it was always present in great numbers at the beginning of the second week of the whoop. The Bordet-Gengou medium with a reaction of P_H 7.2 to 7.4 is favorable for the growth of *B. influenzae*. The colonies develop in from 18 to 24 hours and thus outgrow the *B. pertussis* colonies, for the latter are not apparent until the third or perhaps the fourth day of incubation. The

colonies of *B. influenzae* are very much like those of *B. pertussis*, except that they do not show a light colored area in the blood plate. When, however, the colonies of *B. influenzae* happen to be of the hemolytic variety even this diagnostic difference disappears.

Experimental Work.—In view of the foregoing facts the chief object in the search for a more favorable medium for isolation of the Bordet-Gengou bacillus was to find a substance which would inhibit in a measure the development of the colonies of *B. influenzae* and at the same time be favorable for the growth of *B. pertussis*.

The first attempts at modification consisted in the addition of various dyes, such as gentian violet, methylene blue, methylene green,

TABLE 3
COMPARATIVE GROWTH OF *B. PERTUSSIS* AND *B. INFLUENZAE* AND A MIXTURE OF BOTH ON STRONGLY ACID AND FEEBLY ACID BORDET-GENGOU MEDIUM AND ALSO ON ACID GLYCEROL-VEAL-POTATO AGAR PLUS BLOOD

Plates	Bordet-Gengou Medium		Glycerol-Veal-Potato Agar Plus Blood P _H 6.0	Chocolate Agar P _H 7.2-7.4
	P _H 7.2-7.4	Unadjusted P _H 5.8		
<i>B. influenzae</i>	1.....	++	—	+++
	2.....	±	—	++
	3.....	—	—	+
<i>B. pertussis</i>	1.....	++	+++	—
	2.....	++	++	—
	3.....	±	+++	—
Mixture of <i>B. influenzae</i> and <i>B. pertussis</i>	1 { <i>B. influenzae</i> .. <i>B. pertussis</i> ...	+	—	+++
		+++	+++	++
	2 { <i>B. influenzae</i> .. <i>B. pertussis</i> ...	+	—	±
		++	+++	+
	3 { <i>B. influenzae</i> .. <i>B. pertussis</i> ...	—	—	—
		±	+++	±

brilliant green, carmen borax and others. These were tried in different dilutions with pure cultures of *B. pertussis* and *B. influenzae*. The results were not satisfactory. The majority of these dyes acted alike on these two organisms, while a few even enhanced the growth of *B. influenzae*. Finally, when Grüber's acid green was tried the results were more encouraging. With a dilution of 1:20,000 of this dye, the growth of *B. pertussis* was much better than that of *B. influenzae*. The suggestion of an acid reaction in acid green prompted us to make comparative tests with the 2 organisms on a potato-glycerol-veal agar with the reaction unadjusted, and, therefore, quite acid (P_H 5.8 to 6.1). We are accustomed to use this medium for the growth of *B. mallei*.

Citrated horse blood in proportion of 1:4 was added to the medium at a temperature of 45 C., well mixed with the agar and slanted. *B. pertussis* and *B. influenzae* were inoculated in companion tubes of this medium. The tube inoculated with *B. influenzae* showed almost no growth after 48 hours' incubation. The growth of *B. pertussis*, on the other hand, was astonishing; so much so, that at the first glance, it was thought to be a contamination. Only a smear and a subsequent plating convinced us that we were dealing with a pure culture of *B. pertussis*. On this medium the Bordet-Gengou bacillus grows in a brittle pearly white mass.

The next procedure was to make comparative plate tests of *B. pertussis*, *B. influenzae* and a mixture of both in 4 mediums, 2 of these being unadjusted and therefore of an acid reaction. These mediums were: (1) Bordet-Gengou medium adjusted to P_H 7.2 to 7.4; (2) Bordet-Gengou medium unadjusted, P_H 5.8; (3) potato-glycerol-veal agar plus blood unadjusted, P_H 6, and (4) chocolate agar, P_H 7.2 to 7.4.

Uniform suspensions of the same density (rather light) were made from 48-hour cultures of *B. pertussis* grown on Bordet-Gengou medium and *B. influenzae* grown on chocolate agar; also of a mixture of both organisms. The same sized loop was used for streaking all plates. Further dilution of the suspension was made by inoculating 3 plates in succession with the same loopful.

Table 3 shows how favorably *B. pertussis* grows on the acid mediums as compared with *B. influenzae*; while on the Bordet-Gengou medium adjusted to P_H 7.2-7.4, the *B. influenzae* grows almost as well as *B. pertussis*. On chocolate agar the freshly isolated *B. pertussis* does not grow, while *B. influenzae* gives its usual luxuriant growth.

In the mixture of *B. influenzae* and *B. pertussis* on the acid mediums, the preponderance of *B. pertussis* was made conspicuous by the areas of lighter color surrounding the colonies. There was a good growth of *B. influenzae* on Bordet-Gengou medium with a reaction of P_H 7.2 to 7.4 and much less growth of *B. pertussis*, as indicated by a smaller number of colonies showing the lighter areas on the medium.

The next attempt was to find the limits of acidity favorable for the growth of *B. pertussis*. A series of careful preliminary tests was made to determine the P_H values of the mediums after the addition of different amounts of various acids. It was thought best to use organic acids for this purpose. Small amounts of acetic, lactic and tartaric acids were added to Bordet-Gengou and to potato-glycerol-veal

agar in quantities sufficient to make the final dilutions from 1:2,500 to 1:100. The mediums were then tested as to their reaction.^{15a} These tests were made before the addition of blood, and the reaction may, therefore, have been changed slightly by the blood. On the advice of C. E. Ekroth, potassium oxalate was also tried. Though in itself it is neutral, the medium to which it is added becomes more acid. The results of this test as to acidity are shown in table 4.

It is seen by this table that the same dilutions of the three acids have a more acid effect on the Bordet-Gengou medium than on potato-glycerol-veal agar. This is probably because the Bordet-Gengou medium contains no meat and has therefore practically no buffers.

Thus, while the reaction of potato-glycerol-veal agar after addition of each of the 3 acids in 1:2,000 dilution is from P_H 5.5 to 5.2, the

TABLE 4
REACTIONS OF MEDIUMS ADJUSTED TO DIFFERENT H-ION CONCENTRATIONS BY THE ADDITION OF VARIOUS ORGANIC ACIDS OR OF POTASSIUM OXALATE

Medium	Acid	Final Dilution 1:2,000 P_H	Final Dilution 1:1,000 P_H	Final Dilution 1:500 P_H	Final Dilution 1:100 P_H
Bordet-Gengou.....	Lactic	4.4	4.1	3.7	3.2
Glycerol-potato-veal agar.....	Lactic	5.4	5.0	4.3	3.6
Bordet-Gengou.....	Acetic	4.45	4.4	4.1	3.7
Glycerol-potato-veal agar.....	Acetic	5.3	5.0	4.4	4.1
Bordet-Gengou.....	Tartaric	4.2	3.9	3.6	3.2
Glycerol-potato-veal agar.....	Tartaric	5.5	4.4	4.3	3.6
Bordet-Gengou.....	Potas. oxalate*	4.4	4.5	4.5	4.4
Glycerol-potato-veal agar.....	Potas. oxalate*	4.4	4.5	4.4	4.5

* Only one sample marked "technical, neutral" gave us the reactions recorded in this table. The results in isolation continued to be satisfactory with the dilution of 1:2,000. Later tests of this sample, when added to the Bordet-Gengou medium, showed, however, a much less acid reaction (P_H 6.1 to 6.3) as did also other samples marked "tested purity" and "blue label reagent, neutral."

reaction of Bordet-Gengou medium in the same dilution is from P_H 4.4 to 4.2. In a dilution of 1:1,000, the reaction of potato-glycerol-veal agar is from P_H 5 to 4.4; that of Bordet-Gengou is from 4.4 to 3.9. In 1:500 dilution, the reaction of potato-glycerol-veal agar is from P_H 4.4 to 4.3; that of Bordet-Gengou from 4.4 to 3.6. In 1:100 dilution, the reaction of potato-glycerol-veal agar is from P_H 4.1 to 3.6, while that of Bordet-Gengou is from 3.7 to 3.2. Potassium oxalate, on the other hand, seems to act on both mediums alike, and the reaction is the same (P_H 4.5 to 4.4) in all 4 dilutions.

To prepare these mediums it was found necessary to add the acids in suitable dilution to the medium after sterilization, just before addition

^{15a} The determinations were made by Mr. M. A. Last, chemist in the Division of Media. The colorimetric method was used.

of the blood. If the acids are added to the mediums before the autoclaving, the agar tends to lose its solidifying property. This is true especially when the stronger dilutions of acid are used. Agar containing dilutions of acid as high as 1:2,500 or 1:3,000 can be sterilized with little effect on its firmness.

In making tests as to the limits of acidity favorable for the growth of *B. pertussis*, we confined ourselves entirely to the Bordet-Gengou medium, for reasons which will be given later.

Table 5 shows the limits of growth of pure cultures of *B. pertussis* on Bordet-Gengou medium with the different acids and potassium oxalate. The results as summarized are as follows:

Acetic Acid.—Growth is good on Bordet-Gengou medium with the addition of this acid in 1:2,000 dilution (P_H 4.45); growth also good in 1:1,000 dilution (P_H 4.4); growth is poor in 1:500 dilution (P_H 4.1); no growth occurred at all with 1:100 dilution (P_H 3.7).

TABLE 5
FORTY-EIGHT HOUR GROWTH OF *B. PERTUSSIS* ON BORDET-GENGOU MEDIUM ADJUSTED TO VARIOUS H -ION CONCENTRATIONS WITH DIFFERENT DILUTIONS OF VARIOUS ORGANIC ACIDS OR OF POTASSIUM OXALATE

Acid	Final Dilution 1:2,000 P_H	Growth	Final Dilution 1:1,000 P_H	Growth	Final Dilution 1:500 P_H	Growth	Final Dilution 1:100 P_H	Growth	Control Acid Added Without Blood
Acetic.....	4.45	++	4.4	++	4.1	±	3.7	—	—
Lactic.....	4.4	++	4.1	++	3.7	+	3.2	—	—
Tartaric.....	4.2	++	3.9	++	3.6	+	3.2	—	—
Potassium oxalate..	4.2	++	4.5	++	4.4	++	4.5	++	—

Lactic Acid.—Growth is good with 1:2,000 dilution (P_H 4.4); also good with 1:1,000 (P_H 4.1); fairly good with 1:500 dilution (P_H 3.7); no growth with 1:100 dilution (P_H 3).

Tartaric Acid.—Growth is good with 1:2,000 dilution (P_H 4.2); also good with 1:1,000 (P_H 3.9); fairly good in 1:500 dilution (P_H 3.6); no growth in 1:100 dilution (P_H 3.2).

With potassium oxalate, the growth is good with all 4 dilutions, namely, from 1:2,000 to 1:100 dilution. The primary dilution of potassium oxalate was made by adding 1 gm. to 10 cc of distilled water, which made a 1:10 dilution. The same proportion was observed with tartaric acid.

With pure cultures of *B. pertussis*, the growth on a medium to which acid had been added was, as a rule, good as long as the blood in the medium remained bright red. This was usually the case with the

mediums to which acids in dilutions of 1:2,000 or 1:1,000 were added. When the dilutions were 1:500 and lower, the medium turned brown and little or no growth of *B. pertussis* occurred. With potassium oxalate the medium remained red with all 4 dilutions.

ISOLATION OF *B. PERTUSSIS* IN A SECOND SERIES OF CASES
ON AN ACID MEDIUM

A second series of isolations was undertaken to test the worth of the following mediums for isolation purposes: Potato-glycerol-veal agar plus blood, chocolate agar, Shiga medium and Bordet-Gengou medium.

Potato-glycerol-veal agar plus blood, on which *B. pertussis* in pure culture grows so luxuriantly immediately after isolation, was found not suitable for the actual isolation of the organism. The plates were invariably overgrown, because this medium is too favorable for the growth of the associated organisms of the mouth and nasopharynx.

Chocolate-veal agar had been used first by the writer in 1912-1913 in work on influenza,¹⁶ and later for pertussis;¹⁷ it was then referred to as "coagulated blood veal agar." This medium is not suitable for isolation because *B. pertussis* grows in it only after preliminary cultivation on Bordet-Gengou for a number of weeks, and the organism must become accustomed to the modified blood. After that, the growth becomes luxuriant.

The Shiga medium as described is not suitable because it also is too favorable for the growth of *B. influenzae* and the other organisms usually present in the sputum of pertussis patients.

Bordet-Gengou medium, which is made without meat or peptone, is less favorable for the growth of the associated organisms of the mouth and nasopharynx. This medium of a reaction (P_H 6.1 to 4.4) was the best for purposes of isolation and it was, therefore, the medium selected for our further tests.

The reactions of the mediums for the second series of isolations were as follows:

1. Bordet-Gengou adjusted with normal NaOH to P_H 7.2 to 7.4 (used exclusively in the first series of isolations).
2. Bordet-Gengou unadjusted, P_H 5.8 to 6.1.
3. Bordet-Gengou adjusted with acetic or lactic acids to P_H 5.

¹⁶ Collected Studies from Bureau of Laboratories, Dept. of Health, City of New York, 1912-1913, 7, p. 93.

¹⁷ Arch. Int. Med., 1916, 17, p. 279.

4. Bordet-Gengou with acetic, lactic, tartaric acids in 1:2,000 and 1:1,000 from P_H 4.5 to 3.9.

5. Bordet-Gengou with potassium oxalate in 1:2,000 and 1:1,000 dilutions.

6. Chocolate-veal agar, P_H 7.2 to 7.4.

Of the modifications described under 4, only Bordet-Gengou with lactic acid added in 1:2,000 dilution was retained. As mentioned before, *B. pertussis* grows well in pure culture with each of the 3 acids added in dilutions of 1:1,000 to 1:2,000. This is not true, however, when the same mediums are used for purposes of isolation. It was found that when the acids were added in the proportion of 1:1,000 dilution, all of the plates turned brown the next day and could not be used. When the acids were added in 1:2,000 dilution, the results were variable. In this dilution, the lactic acid proved better than either the acetic or tartaric acids in the same dilution. The most constant results

TABLE 6
ISOLATION OF *B. PERTUSSIS* ON ACID BORDET-GENGOU MEDIUM REACTION P_H 6.1-4.4

Second Series of Twenty-Six Cases		
Number of Cases	Week of Whoop	Number of Isolations
7.....	1	5
7.....	2	3
6.....	3	1
3.....	4	1
3.....	5	0

were obtained with agars containing acetic and lactic acids, P_H 5 (1:2,500 dilution); with potassium oxalate in 1:2,000 and occasionally in 1:1,000 dilutions.

Table 6 shows the superiority of the acid mediums over the medium with a reaction of P_H 7.2 to 7.4. With the Bordet-Gengou medium of the latter reaction in the first series of cases, we succeeded, as stated, in isolating 8 cultures from a total of 35 cases, over 22.8%. In contrast to this figure, our second series of cases tested at the same stages of the disease with the same medium, but of acid reaction, showed over 43.5% isolations (10 in 23 cases).

In the second series, there were really 26 cases including the three in the fifth week of the whoop. Of the 26 cases, 24 came from the home for children. In the majority of cases obtained from the home for children, there were 2 unfavorable factors: The first was the early age of the children, from 2 to 4 years, at which period sputum is obtained with difficulty; the second was the fact that the nature of

the disease was not recognized until the symptoms were more or less advanced and past the catarrhal stage. Under more favorable conditions than these, a still higher percentage of isolations with the acid medium could be expected.

Table 7 gives still further proof of the advantage of an acid reaction. Thus with Bordet-Gengou medium, reaction P_H 7.2 to 7.4, only 4 of 23 cases gave cultures of *B. pertussis*. With the unadjusted Bordet-Gengou (P_H 6.1 to 5.8) 7 of 23; with Bordet-Gengou adjusted to P_H 5 with acetic acid, 10 cultures were obtained. Bordet-Gengou adjusted with potassium oxalate in 1:2,000 dilution (reaction? see note to table 4), 10 positive cultures from 23 cases up to the fourth week of the whoop. As seen in table 6, no results were obtained from any cases in the fifth week of the whoop.

TABLE 7

COMPARATIVE VALUES OF DIFFERENT REACTIONS OF BORDET-GENGOU MEDIUM FOR ISOLATION OF *B. PERTUSSIS* IN DIFFERENT STAGES OF THE DISEASE

Number of Cases	Week of Whoop	Reaction Adjusted with Normal Sodium Hydroxid to P_H 7.2-7.4	Reaction Unadjusted P_H 5.8-6.0	Reaction Adjusted with Acetic Acid to P_H 5.0 (Dilution 1:2,500)	Reaction Adjusted with Lactic Acid to P_H 4.4 (Dilution 1:2,000)	Reaction Adjusted with Potassium Oxalate to P_H 4.5 (Dilution 1:2,000)	Total Number of Isolations
7	1	3	4	5	4	5	5
7	2	1	2	3	2	3	3
6	3	0	1	1	0	1	1
3	4	0	0	1	0	1	1
3	5	0	0	0	0	0	0
Total 23	...	4	7	10	6	10	10

One objection to the acid reaction in the Bordet-Gengou medium is the fact that it is also favorable for the growth of gram-negative cocci. This difficulty is more than compensated for by the control of the growth of *B. influenzae* and other organisms.

In order to ascertain whether *B. pertussis* could be isolated from conditions other than whooping cough, control tests were made, at the suggestion of Dr. W. H. Park. Fifteen sputums from children ill with scarlet fever and measles and various other infections, also three from adults ill with scarlet fever, were tested with the acid Bordet-Gengou medium. No *B. pertussis* was isolated in any case.

In addition to the proportion of isolations, we were interested in ascertaining the length of time during which *B. pertussis* could be obtained from a given case.

Attempts were made in a number of cases to obtain repeated cultures; the sputum was obtained twice, 3 times, and in 1 case 4 times. Positive results were obtained in 2 successive cultures from 2 cases only. The third cultures from the same cases were negative. This work is still in progress.

In Copenhagen, according to Chievitz and Meyer, the children are readmitted to school after the fifth week of the disease after the cultures have proved negative twice, in spite of the fact that the children still continued to whoop and cough. The experiments of these two workers in this direction had convinced them that whooping cough is not infectious after the fifth or sixth week of the disease.

An attempt was made in 2 cases to use the medium mentioned by Inaba, but on account of the meager description given by this writer, we did not know whether we were using the same medium or not. We omitted from the unadjusted Bordet-Gengou medium the glycerol and potato extract. It is evident that such a medium would be even less favorable for the associated organisms than the Bordet-Gengou medium. On the other hand, it may lack sufficient nourishment for the development of *B. pertussis*. This is suggested by the fact that though we obtained positive results on this medium, the colonies were less numerous than on the other mediums used, and on all positive results were given with material from these 2 cases.

The Chievitz and Meyer droplet method mentioned before, was used in six cases of the second series with negative results. These cases were in the second week of the whoop. However, many colonies of *B. influenzae* appeared on chocolate Avery plates from the same cases by the droplet method.

SUBSEQUENT GROWTH OF *B. PERTUSSIS*

After isolation, this organism continues to grow well on Bordet-Gengou medium, especially on the unadjusted P_H 5.8 to 6.1. It grows best on unadjusted potato-glycerol-veal agar (P_H 5.8 to 6.1) plus blood. This medium should be excellent for the cultivation of large quantities of *B. pertussis* and for the making of antigens and vaccines. The medium which is in use at present in our laboratory for this purpose is chocolate-veal agar. Although *B. pertussis* grows luxuriantly on this medium, it does not do so until many weeks after isolation, as mentioned before; while on potato-glycerol-veal agar plus blood it grows at once.

B. pertussis grows just after isolation also on Shiga's medium but not as luxuriantly as on the potato-glycerol-veal agar plus blood or on chocolate veal agar, after preliminary cultivation.

SUMMARY

It was found that a definitely acid reaction in a suitable medium is favorable for the isolation and growth of *B. pertussis*. Such a reaction is especially valuable because it inhibits the growth of *B. influenzae* and other organisms found in the sputum of patients with pertussis.

The most favorable point of acidity for isolation was found to be at P_H 5.

The limits of acidity favorable to the growth of *B. pertussis* are P_H 6.1 to 4.4.

The unadjusted Bordet-Gengou medium for the cultivation of *B. pertussis* is usually of the reaction P_H 5.8 to 6.1. If a more acid reaction is desired, this can be obtained by adding one of several organic acids to a medium, preferably after the latter has been sterilized and before the addition of the blood.

B. pertussis grows immediately after isolation most luxuriantly on potato-glycerol-veal agar unadjusted (P_H 5.8 to 6.1) plus blood in proportion of 1:3 or 1:4 added at a temperature of 45 C.; but this medium is too favorable for the growth of various other organisms found in the sputum and is therefore not suitable for the isolation of *B. pertussis*.

On the other hand, the Bordet-Gengou medium, which contains neither meat nor peptone, is less favorable for the growth of the associated organisms of the mouth and nasopharynx. It was found, therefore, that this medium after adjustment to a suitable acid reaction (P_H 5) was the best for the isolation of *B. pertussis*.

THE EXISTENCE OF MORE THAN ONE IMMUNOLOGIC TYPE OF B. PERTUSSIS

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In an investigation of the antigenic properties of pertussis vaccine, it became necessary to isolate a new strain of *B. pertussis* for comparison with older stock strains. Several strains, therefore, were isolated having all the typical morphologic and cultural characteristics of the Bordet-Gengou bacillus. These strains were tested agglutinatively with the serum of a rabbit which had been inoculated with the old stock strain utilized in the vaccine experiments. This serum agglutinated the freshly isolated strains nearly as well as the homologous serum strain. Rabbits were then injected with a suspension of one of the newly isolated strains. This serum to our surprise caused only slight agglutination (1:100) of the old stock strain, whereas the other freshly isolated strains were agglutinated to practically the same degree as the serum strain.

These observations led to a more detailed study of a series of strains of *B. pertussis*, utilizing agglutination and agglutinin-absorption for differentiation. From various sources we collected a series of stock strains among which was a strain with a history of having been obtained from Bordet. Fresh strains were obtained from 17 cases. For most of these strains we are indebted to Dr. Olga R. Povitzky, who isolated them while working on the improvement of mediums for the isolation and cultivation of *B. pertussis*.

The agglutination and agglutinin-absorption technic follows:

Agglutinating serums were prepared by injecting rabbits either intravenously or intraperitoneally with suspensions of *B. pertussis*. The injections were made on each of 3 successive days and followed by a rest period of 4 days. As a rule 5 series of injections were required. The dose varied from about 1/10th chocolate agar slant to a maximum of about 1 full slant. "Chocolate agar" is hot veal-agar (95 C.) to which 10% of citrated horse blood is added.

Agglutination antigens were prepared by scraping the 48-hour growth from chocolate agar and making a suspension in salt solution containing 0.1% of formaldehyde solution, 40%. The suspension was shaken by hand and filtered

through a thin layer of absorbent cotton, diluted to an arbitrary standard of opacity and kept in the ice chest. Freshly prepared antigens did not agglutinate as well as those kept for 48 hours or longer. In carrying out the agglutinations 0.9 c.c. of antigen and 0.1 c.c. of the serum dilution were employed. The tests were incubated at 56 C. for 18 hours and then read.

The agglutinin-absorption was carried out as follows: The absorbing dose of bacilli was obtained by centrifuging either a freshly prepared suspension of bacilli or a suspension in formalin-salt solution which had been stored in the ice chest. After the bacillary mass was sedimented, the supernatant fluid was discarded and the appropriate amount of serum and salt solution added. The bacilli were resuspended in the diluted serum, incubated at 45 C. for 3 hours and then placed in the ice chest over night. The following morning the mixture was centrifuged until clear, and the supernatant diluted serum was used for the tests. The controls consisted of diluted serum without addition of the bacillary mass or with the addition of a strain which had been found negative in regard to absorption.

The approximately minimum absorbing dose was determined for the serums used. This ranged between 1:100 to 1:200 for the different serums, the absorbing dose being expressed in terms of volume of bacterial mass to volume of added serum diluted 1:10.

When agglutinative differences were first noted, a few preliminary absorption tests were carried out with an arbitrary absorption dose of 1:10. This yielded a difference which correlated with the differences obtained on direct agglutination. Although titration showed that this absorbing dose was 10 or more times greater than was necessary, its use was continued for the preliminary separation of the cultures.

The agglutinative and agglutinin-absorption results with a series of strains are given in table 1. As may be seen, the strains tested fall into 2 groups, A and B.

These results show the tendency of group B (represented by the "Bordet" strain) to cross agglutination with serum 253 and the relative absence of reciprocal agglutination of group A (represented by strain 253) with the "Bordet" serum.

The same general tendency is noted in the absorption results. Absorption of serum Bordet (group B) by the individual strains in group A resulted in no appreciable reduction in the specific agglutinins in spite of the excessive absorbing dose employed.

Absorption of the serum 253 (group A) by the members of group B resulted in a relatively marked reduction of the specific agglutinins. In one instance, strain 98 P.D., there was an apparently complete removal of the specific agglutinins. In this case a smaller absorbing dose (1:80) showed that this strain was distinctly different from the serum strain 253. Although the results show the disadvantage of an

excessive absorption dose, its use in this instance has resulted in not only showing the relationships of the 2 groups, but also their essential difference.

Because of the results obtained with serum 253 (group A), a serum for one of the other strains in this group was tried the same method

TABLE 1
PRELIMINARY GROUPING BASED ON THE RESULTS OBTAINED WITH A LARGE ABSORBING
DOSE 1:10

Strains Tested	Serum 253			Serum Bordet P. D.		
	Agglutination			Agglutination		
	Before Absorption	After Absorption		Before Absorption	After Absorption	
		Absorbing Strain	Serum Strain		Absorbing Strain	Serum Strain
Group A						
253.....	2,000++	0	0	0	0	1,000++
M 254,1.....	1,500++	0	0	100++	0	2,000++
217 L.....	1,000++	—	—	0	0	1,000++
246 L.....	1,000++	—	—	0	0	1,000++
247 L.....	500++	—	—	0	0	1,000++
248.....	1,500++	0	0	0	0	1,000++
250 L.....	1,000++	—	—	0	0	1,000++
251.....	1,500++	0	0	0	0	1,000++
252 M.....	1,500++	—	—	0	0	2,000++
Group B						
Bordet P. D.	1,000++	0	200++	2,000++	0	0
0590 P D.....	1,000++	0	200++	1,000++	0	0
98 P D*.....	800++	0	0	1,000++	0	0
110 P D.....	800++	0	100++	1,000++	0	0
163 P D.....	800++	0	400++	1,000++	0	0
248 P D.....	500++	0	200++	1,000++	0	0
248 M.....	800++	0	400++	1,000++	0	0
M 254,4.....	1,000++	0	150++	1,000++	0	0
M 255,4.....	800++	0	400++	1,000++	0	0
M 256,11.....	800++	0	200++	1,000++	0	0
P 257,5.....	1,000++	0	100++	1,000++	0	0

* In dose of 1:80 no agglutination of absorbing strain in dilution of 1:25, but marked agglutination of serum strain at 1:400.

In this and the following tables, "Bordet" is a strain obtained from Bordet in 1911.

The numbers below 254 refer to strains isolated in this laboratory 1913-14; letters after the numbers designate laboratories from which the strains, originally sent out from this laboratory, were obtained. Strain 0590 was isolated about 1911.

Strains 254 and higher were isolated between February, 1921, and April, 1922, by Povitzky (P) and Mishulow (M); the number after the comma refers to individual fishings.

In all tests 1:100 is the lowest dilution used in the direct agglutination tests; 1:25 is the lowest dilution used after absorption, and in all cases in which there was no agglutination in this dilution, higher dilutions were without effect also; 1:200++ etc., indicates highest dilution given marked but partial agglutination.

+++ , complete agglutination; ++ , marked but partial agglutination; + , slight agglutination; 0 , no agglutination; — , not tested.

being used. The results with this serum 251 (group A) are given in table 2. The same tendency to reduction of the specific agglutinins when absorbed by members of group B is evident. Comparison with the results in table 1 show, however, that the results with individual strains vary with the serum employed. Thus in table 2, 98 P.D. is not noteworthy for the degree of reduction obtained with the 1:10

dose; whereas with the same sized dose this strain shows in table 1 an apparent complete absorption of the agglutinins from a serum for the heterologous group. The different degrees of absorption shown by M. 255, 4, are also marked.

The complete absorption of serums 253 or 251 (group A) when absorbed by members of group A, or the similar results with serum B absorbed by the strains of group B (tables 1 and 2), could not be taken as evidence of the likeness of the strains within each group, primarily, because of the large absorbing dose employed.

Further tests of the serums were carried out, therefore, using an absorbing dose so small that it would surely elicit differences should they exist. The cultures already noted in table 1 as well as other

TABLE 2
CONFIRMATORY RESULTS OBTAINED WITH ANOTHER SERUM, USING A LARGE ABSORBING
DOSE 1:10

Strains Tested	Serum 251: Agglutination		
	Before Absorption	After Absorption	
		Absorbing Strain	Serum Strain
251.....	4,000 ++	0	0
253.....	4,000 ++	0	0
M 254,1.....	6,000 ++	0	0
0590 P D.....	1,000 ++	0	3,000 ++
98 P D.....	1,000 ++	0	3,000 ++
110 P D.....	2,000 ++	0	3,000 ++
163 P D.....	1,000 ++	0	3,000 ++
M 254,4.....	600 ++	0	1,500 ++
M 255,4.....	2,000 ++	0	100 ++
M 256,11.....	1,000 ++	0	1,500 ++

Other confirmatory tests were made with serum M 255,4, testing 16 strains, and with serum M 254,4, testing 11 strains. The results with these serums were so closely like those obtained with serum Bordet (table 1) that there seems to be no reason for publishing the tabulation of the results.

strains were tested in this way (tables 3 and 4). The results indicate that the individual strains within each of the 2 groups are alike. It should be noted that the absorbing dose was in some instances less than the minimum required to give complete absorption within the range of dilutions employed. The use of this small dose rather than a multiple of the average minimum absorbing dose was intentional as we wished to obtain the slightest indications of differences, if possible, as a guide to the selection of other strains to be used for the preparation of other antisera. In the instances in which a residuum of agglutinins remains after absorption, the action on the absorbing strains and on the serum strain are so nearly alike as to give no such indication. Where there is a difference, it is so slight as to be negligible. In only

one instance, strain 247 L in table 4, is there an appreciable difference; this however, is directly attributable to the relative inagglutinability of this strain.

The results given clearly demonstrate the existence of two groups and strongly indicate the likeness of the strains within each group. There remains, however, the possibility of demonstrating differences

TABLE 3
ABSORPTION OF SERUM "BORDET" GROUP B. BY STRAINS OF GROUP B., ABSORBING DOSE, 1:200

Strains Tested	Agglutination Before Absorption	Agglutination After Absorption	
		Absorbing Strain	Serum Strain
Bordet P D.....	2,000 +	0	0
0590 P D.....	1,000 ++	0	0
98 P D.....	2,000 +	0	0
110 P D.....	2,000 +	0	0
163 P D.....	2,000 +	0	0
248 P D.....	1,000 ++	0	0
248 M.....	1,000 ++	0	0
249 L.....	1,000 +	0	0
M 254,3.....	1,000 ++	0	0
M 255,1.....	1,000 +++	0	0
M 256,1.....	1,000 ++	0	0
P 257,5.....	2,000 +	0	0
P 258,1.....	1,000 ++	0	0
P 259,1.....	2,000 +	25 +	0
P 260,1.....	2,000 +	0	0
P 261,1.....	1,000 ++	0	0
P 262,8.....	2,000 +	25 ++	25 ++
P 263,5.....	2,000 +	25 +	25 +
P 264,4.....	2,000 +	25 +	25 +

TABLE 4
ABSORPTION OF SERUM 253 GROUP A BY STRAINS OF GROUP A. ABSORBING DOSE, 1:200

Strains Tested	Agglutination Before Absorption	Agglutination After Absorption	
		Absorbing Strain	Serum Strain
253.....	2,000 +	50 +	50 +
M 254,1.....	2,000 +	25 +++	50 +
217 L.....	1,000 ++	0	0
246 L.....	1,000 ++	0	25 +
247 L.....	400 +++	0	50 +
248.....	1,000 +++	25 ++	25 ++
250 L.....	1,000 +++	25 ++	25 ++
251.....	2,000 +	25 ++	25 ++
252 M.....	1,000 +++	25 ++	25 ++

within each group by the use of serums for strains other than those already employed. For instance, although strain M 254, 4, might absorb the agglutinins from the "Bordet" serum, the "Bordet" strain might not be able to absorb the agglutinins from a serum for strain M 254, 4. Failing to obtain any suggestion in the results given in tables 3 and 4, we have gone no further than to test the reciprocal absorption

of the serums already prepared. In these tests the approximately minimal absorbing dose was employed. The results may be briefly summarized as follows: Group A.—251 = 253, on reciprocal absorption; 251 = M 254, 1, on reciprocal absorption; 253 = M 254, 1, on reciprocal absorption. Group B.—“Bordet” = 255, 4, on reciprocal absorption; Bordet = M 254, 4, on reciprocal absorption; M 255, 4 = M 254, 4, on reciprocal absorption.

Because these results did not suggest the probability of a subdivision within the groups, further serums were not prepared.

TABLE 5
TABULAR SUMMARY OF STRAINS FROM INDIVIDUAL CASES

Cases	Number of Fishings Tested	Grouping			
		Group A		Group B	
		Classified on the Basis of Direct Agglutination Only	Classified on the Results of Agglutination and Absorption	Classified on the Basis of Direct Agglutination Only	Classified on the Results of Agglutination and Absorption
M 254.....	4	0	1	0	3
M 255.....	20	0	0	14	6
M 256.....	21	0	0	16	5
P 257.....	2	0	0	0	2
P 258.....	15	0	0	11	4
P 259.....	7	0	0	4	3
P 260.....	4	0	0	2	2
P 261.....	2	0	0	1	1
P 262.....	35	0	0	26	9
P 263.....	15	0	0	11	4
P 264.....	1	0	0	0	1
P 265.....	1	0	0	0	1
P 266 a+.....	1	0	0	0	1
P 266 b+.....	1	0	0	0	1
P 267.....	3	0	0	2	1
P 268 a+.....	3	0	0	2	1
P 268 b+.....	3	0	0	2	1
P 269.....	3	0	0	3	0
P 270.....	3	0	0	3	0

a+ and b+ = successive isolations from the same case with an interval of two weeks.

As is noted in table 1, both type A and type B were isolated from case 254. This led us, when possible, to make numerous fishings from the plates inoculated with the sputum from the cases of pertussis which came to us later. These fishings have been tested and grouped either by agglutination alone or by agglutination and agglutinin-absorption. The number of cases and fishings per case thus tested are noted in table 5. All the cases with the exception of 254 have yielded only strains of group B.

The grouping of all the strains studied may be summarized thus: Among the old stock strains, both types were encountered. In some instances, transplants of the same cultures carried by different labora-

tories have been found to be different. This must be due either to mislabeling or to the fact that the original strains contained a mixture of the types. In one instance there was apparently a mixture in one of the stock cultures. From the recent cases, one case yielded both types, but all the other cases have yielded only type B. What interpretation is to be made of these results it is impossible to state. It may be that the different types may vary in their prevalence at different times; or it may be suggested that only one of the types is a true *B. pertussis*. This cannot be disproved, but the presumption is against this conclusion in view of the morphologic and cultural similarity as well as the agglutinative relationship. It may be argued that the differences are due to a change of serologic characteristics among some of the stock strains. This argument cannot be considered in view of the isolation of both types from a recent case (254).

Previous workers have utilized the agglutination reaction in the study of *B. pertussis*. Klimenko,¹ Wollstein² and Odaiva³ found no agglutinative differences among the strains that they isolated. Povitzky⁴ studied 27 strains and concluded that they belonged to one group. This failure to obtain differences was probably due to the use of a serum obtained by the injection of 3 strains. Giese⁵ observed agglutinative differences between 2 strains having the morphologic and cultural characteristics of *B. pertussis*. He did not follow up this suggestive observation.

In view of the report of Bordet and Sleeswyk⁶ that they could influence the antigenic characters of *B. pertussis* by the use of different mediums, all our serologic tests were carried out with bacilli cultivated on one type of medium, namely, chocolate agar. Bordet and Sleeswyk reported that if they immunized a horse or a rabbit with bacilli grown on a blood containing medium, such as the Bordet-Gengou medium, that the resulting agglutinins were active against bacilli grown on the same medium, but less active against bacilli grown on plain agar. The agglutinins could be completely absorbed from such a serum by bacilli grown on the Bordet-Gengou medium but not by the same strain grown on plain agar. If a horse or a rabbit were injected with bacilli grown on plain agar, the results were reversed. If the strain grown on plain

¹ Arch. d. Sc. Biol., 1910, 15, p. 25.

² Jour. Exper. Med., 1909, 11, p. 41.

³ Centralbl. f. Bakteriol., I. O., 1911, 61, p. 239.

⁴ Arch. Int. Med., 1916, 17, p. 1279.

⁵ Ann. de l'Inst. Pasteur, 1918, 32, p. 522.

⁶ Ibid., 1910, 24, p. 476.

agar were again cultivated on Bordet-Gengou medium, its agglutinative characteristics became the same as the strain continuously grown on a blood medium.

These results have never been confirmed to our knowledge. Povitzky found that a horse injected with two strains grown on Bordet-Gengou yielded a serum during the earlier period of immunization which agglutinated bacilli grown on the same medium but not those grown on chocolate agar. After a longer period of immunization no such differences were noted. Working with rabbit serums she found no

TABLE 6

THE INFLUENCE OF THE MEDIUM ON AGGLUTINATION AND AGGLUTININ-ABSORPTION; SERUM BORDET, FROM RABBIT IMMUNIZED WITH BACILLI GROWN ON CHOCOLATE AGAR

Agglutination Before Absorption			Agglutination After Absorption				
Strain	Grown On	Titer	Absorbed By		Tested With		Titer
			Strain	Grown On	Strain	Grown On	
Bordet	Chocolate agar	3,000 ++	Bordet	Chocolate agar	Bordet	Chocolate agar...	0
						Povitzky.....	0
						Bordet-Gengou...	0
						Agar.....	0
Bordet	Povitzky	3,000 +	Bordet	Povitzky	Bordet	Chocolate agar...	0
						Povitzky.....	0
						Bordet-Gengou...	0
						Agar.....	0
Bordet	Bordet-Gengou	3,000 ++	Bordet	Bordet-Gengou	Bordet	Chocolate agar...	0
						Povitzky.....	0
						Bordet-Gengou...	0
						Agar.....	0
Bordet	Agar	3,000 +	Bordet	Agar	Bordet	Chocolate agar...	0
						Povitzky.....	0
						Bordet-Gengou...	0
						Agar.....	0
P 264,4	Chocolate agar	3,000 ++	P 264,4	Chocolate agar	P 264,4	Chocolate agar...	0
						Povitzky.....	800 +
						Bordet-Gengou...	800 +
P 264,4	Povitzky	1,600 +	P 264,4	Povitzky	P 264,4	Chocolate agar...	400 +
						Povitzky.....	400 +
						Bordet-Gengou...	800 +
P 264,4	Bordet-Gengou	3,000 +	P 264,4	Bordet-Gengou	P 264,4	Chocolate agar...	100 +
						Povitzky.....	800 +
						Bordet-Gengou...	800 +

agglutinative or absorptive differences in regard to the mode of cultivation of the bacilli used.

We have made comparative tests with serums obtained from rabbits injected with *B. pertussis* grown on chocolate agar. Antigens were prepared from the bacilli grown for from 5 to 20 generations on plain agar, chocolate agar, Bordet-Gengou medium containing about 25% of unheated blood, and a medium studied by Povitzky. This medium was a potato-glycerol-veal agar which contained about 35% of unheated blood.

Table 6 is representative of the results obtained. With the older stock strains, no appreciable differences in agglutinative or absorptive characteristics were noted. With the more recently isolated strains, which, however, could not be grown on plain agar, considerable differences were observed. The antigens grown on chocolate agar (heated blood) agglutinated well and showed a high absorptive capacity. Those from mediums containing unheated blood (either Bordet-Gengou or Povitzky) agglutinated poorly or in some instances not at all and had a low absorptive capacity. A transfer for even one generation to chocolate medium resulted in a complete return to the higher level of agglutinability and absorptive capacity.

TABLE 7
THE INFLUENCE OF THE MEDIUM ON AGGLUTINATION AND AGGLUTININ-ABSORPTION; SERUM
M 255 FROM RABBIT IMMUNIZED WITH BACILLI GROWN ON
BORDET-GENGOU MEDIUM

Agglutination Before Absorption			Agglutination After Absorption				
Strain	Grown On	Titer	Absorbed By		Tested With		Titer
			Strain	Grown On	Strain	Grown On	
M 255,4	Chocolate agar	2,000 ++	M 255,4	Chocolate agar	M 255,4	Chocolate agar...	0
						Bordet-Gengou...	800 +
M 255,4	Bordet-Gengou	1,000 +	M 255,4	Bordet-Gengou	M 255,4	Chocolate agar...	50 ++
						Bordet-Gengou...	800 +
P 270,3	Chocolate agar	2,000 +	P 270,3	Chocolate agar	P 270,3	Chocolate agar...	0
						Bordet-Gengou...	400 +
					M 255,4	Chocolate agar...	0
						Bordet-Gengou...	800 +
P 270,3	Bordet-Gengou	800 +	P 270,3	Bordet-Gengou	P 270,3	Chocolate agar...	50 ++
						Bordet-Gengou...	400 +
					M 255,4	Chocolate agar...	0
						Bordet-Gengou...	1,000 +

A serum from a rabbit injected with a recently isolated strain grown on the Bordet-Gengou medium was tested in the same way. This serum gave much better agglutination with bacilli grown on the chocolate medium. When the serum was absorbed by bacilli grown on the chocolate medium, the agglutinins for the chocolate agar grown bacilli were removed; when absorbed by bacilli grown on the Bordet-Gengou medium, these agglutinins were only reduced. In neither instance, however, was there an appreciable reduction in the "agglutination" of the Bordet-Gengou antigen. These results are given in table 7. The fact that the absorption was not followed by a reduction in the degree of clumping of the Bordet-Gengou antigen, raised the question whether the flocculation with this type of antigen was due to specific agglutination.

The 2 types of antigens of the freshly isolated strain were tested, therefore, with 2 normal rabbit serums; also with 2 homologous serums, one from a rabbit injected with bacilli grown on the Bordet-Gengou medium, the other from a rabbit injected with bacilli grown on chocolate agar. Both the immune serums agglutinated the chocolate-agar antigens, whereas, the normal serums did not. All the serums, including the normal, "agglutinated" the antigens prepared on Bordet-Gengou medium (horse blood) equally well. Antigens grown on a Bordet-Gengou medium containing rabbit blood were not "agglutinated" by the normal serums but showed a slight reaction with the immune serums. At first glance this would seem to indicate that the clumping of the Bordet-Gengou (horse blood) antigen was a precipitation reaction between rabbit and horse serum. Antigens of old stock strains, however, did not show the same results. As has been noted in the foregoing, the agglutinability and absorptive capacity of the older stock strains were not influenced by the medium on which they were grown. Why only the freshly isolated strains should be influenced by the medium and why only the antigens of such strains grown on unheated blood mediums should give nonspecific clumping, remains to be ascertained. These results again emphasize the dangers of employing antigens obtained from mediums containing unheated blood or serum. The recent report of Kelly,⁷ who found similarly bizarre reactions in studying the precipitin reaction in gonorrheal infections, is of especial interest in this connection.

The results we have obtained do not in any way confirm the findings of Bordet. The observation of Povitzky that the antigens of recently isolated strains grown on the Bordet-Gengou medium were agglutinated much better during the earlier period of immunization is apparently explained by our observations; that is, the reaction was due to the horse serum without regard to its content of specific agglutinins.

SUMMARY

A series of strains having the typical morphologic and cultural characteristics of *B. pertussis* have been studied by means of agglutination and agglutinin-absorption. These tests have demonstrated that the cultures studied fell into two serologic groups. These groups are designated as A and B. Antiserums for group B agglutinate the strains of group B but agglutinate the strains of group A slightly or not at all. Group A serums, however, agglutinate not only the strains of group A,

⁷ Jour. Infect. Dis., 1922, 30, p. 623.

but also agglutinate the strains of group B to a considerable extent. The absorption of group B serums by strains of group A does not appreciably reduce the content of agglutinins for strains of group B. The absorption, however, of group A serums by strains of group B results in a reduction in the agglutinins for strains of group A. The serologic differences are therefore sharply defined in one direction, but group relationship is shown in the reverse direction. The strains within each group are alike as far as is indicated by the absorptions of serums for different members within the same group.

The antigens for these tests were all prepared from the same medium; the serologic differences cannot be attributed, therefore, to the influence of different mediums. The growth on blood mediums influences the agglutinative characteristics of freshly isolated strains of *B. pertussis* but we have been unable to confirm Bordet's observations on this point.

The existence of two groups of *B. pertussis* is of immediate interest because of their possible bearing on the use of pertussis vaccine.

With the exception of one case of whooping cough which showed the presence of both types, all the recent cases have yielded only strains of group B.

FURTHER NOTES ON EXPERIMENTAL MEASLES IN RABBITS AND MONKEYS

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During the past winter confirmation of our work on experimental measles in rabbits and monkeys¹ was undertaken. Blood from 3 early cases of measles was used for the inoculation of 2 series of animals. The blood was drawn on the second day of the disease from patients with measles in the Willard Parker Hospital for Contagious Diseases and within an hour was injected into the animals.

CASE HISTORIES

Patient K: Ill 2 days, coryza, conjunctivitis, cough, Koplik spots, general maculopapular rash.

Patient Q: Same symptoms as patient K.

Patient Z: Ill 2 days, coryza, purulent conjunctivitis, Koplik spots, general maculopapular rash.

Series 1.—The blood of patients K and Q was drawn on the same day, pooled and inoculated into 1 monkey and 3 rabbits (chart 1). The inoculations were intravenous and citrated blood was used.

Monkey 31 (*M. rhesus*) received 12 c c of blood and developed a leukopenia on the fourth day; this was followed on the next day by an enanthem. Then Koplik spots developed and on the sixth day a general maculopapular rash was noted. A fine branny desquamation began on the ninth day.

Three rabbits (1422, 1423, 1425) inoculated with 3.5, 4 and 5 c c, respectively, developed reactions on the fourth and fifth days. The reaction consisted of lacrimation, erythema and exanthem followed by desquamation. One of these rabbits (1422) was bled on the eighth day after inoculation and 3 rabbits (1432, 1433, 1438) were inoculated with 4, 6 and 7 c c, respectively, of the citrated blood. On the second day rabbit 1438 developed an enanthem which lasted for 4 days. The entire labial mucosa was hyperemic, the lips thickened and swollen and the lower labial mucosa intensely injected. No Koplik spots were noted. The exanthem appeared on the eighth day and was

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¹ Jour. Infect. Dis., 1921, 29, 4, p. 429.

followed by a flaky desquamation. No enanthem was noted in the other 2 rabbits, but the exanthem which appeared on the fourth and seventh days was marked. A general maculopapular rash was present on the chest, axillae, and groin; brownish pigmentation and large flaky desquamation followed on the eleventh day.

Two rabbits (1444, 1448) were inoculated with 6 and 7 c c, respectively, of citrated blood drawn from rabbit 1438 on the eighth day. Both of these animals developed reactions and rabbit 1444 was bled on the fifth day.

Blood from Measles Patients K and Q

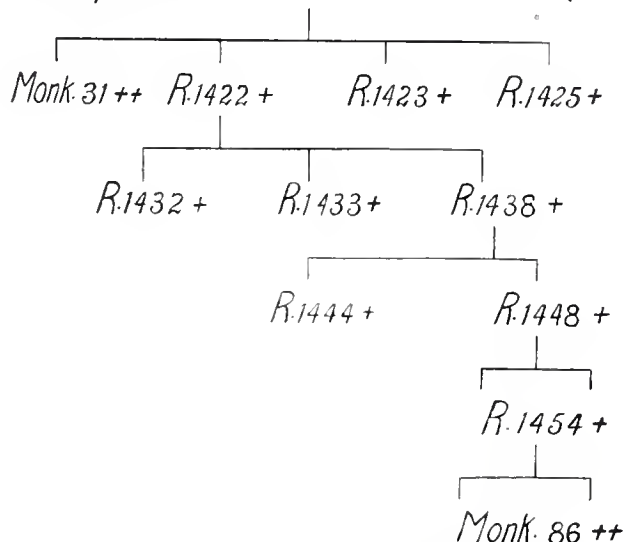


Chart 1.—Transmission of measles virus from measles patients K and Q through four rabbits to a monkey. Suggestive reaction +; typical reaction ++.

Rabbit 1454 inoculated in turn with 10 c c of this blood showed a marked erythematous rash on the third day. From this animal, 10 c c of blood was drawn on the fourth day and inoculated into monkey 86 (M. rhesus). A leukopenia occurred on the fourth day. On the following day an enanthem was noted on the upper and lower labial mucosa. No other symptoms were noted until the eighth day when a marked exanthem appeared on the face, neck and shoulders; the rash spread to the abdomen and groin, and gradually faded; desquamation commenced on the tenth day.

As seen in chart 1, these typical symptoms in the monkey developed after four rabbit passages.

Series 2.—Measles virus from the blood of patient Z (chart 2) was successfully carried through 3 rabbits and 3 monkeys (*M. rhesus*) without any apparent diminution in its ability to produce typical measles symptoms in the monkey. Our idea in passing the virus from monkey to monkey at the end of the experiment was to eliminate as far as

Blood from Measles Patient Z

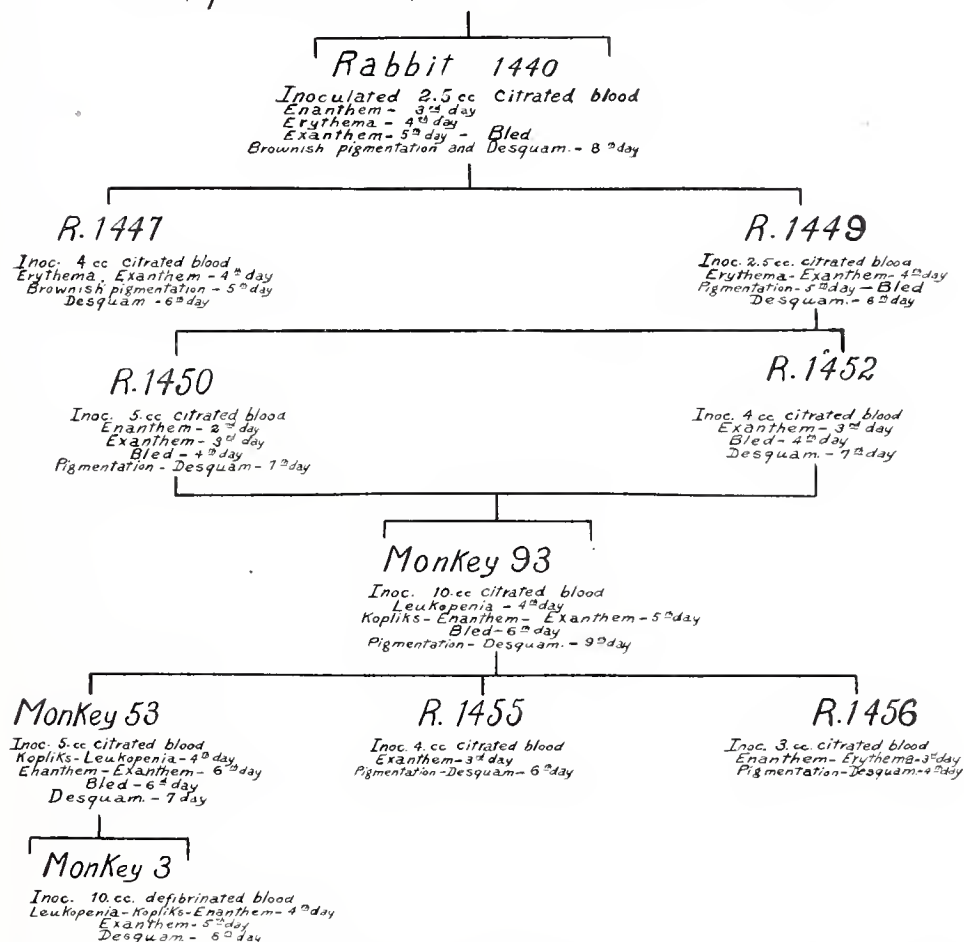


Chart 2.—Transmission of measles virus from measles patient Z through three rabbits and three monkeys.

possible all question of a serum rash caused by the injection of blood from a different species of animal.

Citrated blood in amounts varying from 2.5 to 10 cc was used throughout with the exception of the last passage to monkey 3 when defibrinated blood was used. All inoculations were intravenous. The symptoms are recorded in the chart.

In order to note the appearance of the rash, all of the rabbits in both series were shaved the day before injection. Our previous experience had shown that careful shaving of rabbits produces no lasting irritation which would be confused with a true erythematous rash. White blood counts were made on all animals. In the case of the rabbits, the results confirmed our previous findings that the white blood count of these animals is inconstant and cannot be relied on as a diagnostic point in rabbits.

Controls.—In our previous work we had used 11 rabbits as controls giving them intravenous injections of blood from diseases other than measles (diphtheria, lobar pneumonia, lethargic encephalitis), also normal human blood and normal rabbit blood. As none of these rabbits showed any reaction to these injections, we did not deem it necessary to repeat this part of the work.

A normal monkey was, however, injected intravenously with 5 c c of normal defibrinated human blood and gave no reactions to the injection. Daily blood counts on this animal showed no leukopenia nor did an enanthem or exanthem develop.

SUMMARY

In the first series of animals the virus of measles obtain from patients with measles on the second day of the disease was passed through 4 rabbits and then produced symptoms typical of measles in a monkey.

In the second series of animals the virus obtained as stated was passed through 3 rabbits and then through 3 monkeys in which symptoms typical of measles were produced. The monkey to monkey passage eliminated any question of rash due to foreign protein.

A control monkey injected with normal human blood showed no reaction.

CONCLUSION

The results of these two series of passages confirm our previous findings that the virus of measles survives rabbit passage and produces symptoms typical of measles in the monkey (*M. rhesus*).

SEROLOGIC RELATIONSHIPS IN THE STREPTOCOCCUS VIRIDANS GROUP

INFLUENZA STUDIES XI

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A variety of serologic reactions has been used in attempts to group strains of *Streptococcus viridans*. Floyd and Wolbach¹ reported that agglutination and complement-fixation tests gave groupings which paralleled those obtained by sugar reactions. Kligler's² results, however, with agglutination were far less definite. Kinsella and Swift,³ using complement fixation, recognized 3 groups of nonhemolytic streptococci, but Clawson,⁴ after studying both of these reactions on 150 strains of *Streptococcus viridans*, concluded that nonhemolytic streptococci were heterogeneous in character. Howell⁵ reported that complement-fixation tests were useless in correlation with hemolysis, fermentation reactions or source of strains, except that strains from definite "disease groups" "tended toward group specificity." Evidence for this is also found in the work of Howell and Anderson⁶ with the complement-fixation test in influenza, of Tunnicliff⁷ with opsonic tests on green-producing streptococci from the same disease, of Rosenow⁸ with agglutination tests in influenza, of Herrold⁹ with agglutination and absorption tests on streptococci from chronic prostatic infections, and to a less extent in that of Hodge and Cohen¹⁰ in a study of streptococci found in the sputum of patients suffering from bronchial asthma. The latter workers found certain strains which were agglutinated by a number of serums but which in turn did not produce agglutinins for the homologous strains of these serums. All the evidence obtainable points toward the production of group antibodies by these streptococci.

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¹ Jour. Exper. Med., 1914, 20, p. 72.

² Jour. Infect. Dis., 1915, 16, p. 327.

³ Jour. Exper. Med., 1917, 25, p. 877.

⁴ Jour. Infect. Dis., 1920, 26, p. 93.

⁵ Ibid., 1918, 22, p. 230.

⁶ Ibid., 1919, 25, p. 1.

⁷ Ibid., 1920, 26, p. 405.

⁸ Jour. Am. Med. Assn., 1919, 72, p. 1609.

⁹ Jour. Infect. Dis., 1922, 30, p. 80.

¹⁰ Ibid., 1922, 30, p. 400.

The results of Gordon¹¹ in grouping pathogenic hemolytic streptococci by the use of agglutinin absorption suggested that this reaction might be applied to the *Streptococcus viridans* group, and I have attempted to do this in a systematic way.

The strains studied were obtained from the throats of 6 normal subjects and from the feces of the same persons. They all belonged to the group designated as *Streptococcus viridans*: that is, they were gram-positive cocci occurring in chains of varying length, fermenting dextrose and lactose with the production of acid, producing methemoglobin when grown on blood agar and being insoluble in bile. A total of 46 strains have been studied. Many more were originally isolated but were lost before the work was completed. The throat strains were difficult to keep alive whereas the fecal strains retained their viability with little trouble. Of the 46 strains on which absorption tests were finally made, 14 were from the throat and 32 from the feces. Of the 14, three fermented mannite and 7 salicin (including 1 mannite fermenter). Of the 32 fecal strains 12 fermented mannite and 25 attacked salicin, including all but 1 of the mannite fermenters. None of the 46 strains attacked inulin. Animal tests showed that the strains were nonpathogenic—1 agar slant injected intraperitoneally into a white mouse having no effect.

To obtain the immune serums 7 strains were selected at random and intravenous injections made into rabbits. Three consecutive daily doses of dead organisms were first given; the animal was then allowed to rest 4 days, and then injected with live organisms for 3 consecutive days, and so on. The suspension of organisms for immunization was made from a 24-hour growth in dextrose broth by centrifuging, washing once with salt solution and finally suspending in salt solution. After 3 weeks the animals were bled from the heart, allowed to rest 1 week and injected with live organisms as before. The process may be continued indefinitely. The agglutinin content of the serums was often very high, particularly after 3 series of injections. The titer of the serums actually used in the absorption tests ranged from 1:400 to 1:80,000, as tested against the homologous strains. Toward other strains, even those isolated from the same source at the same time, the titer was not usually so high. Of the 7 serums prepared, 4 proved to be identical. Of these, 2 were made with cocci from the throat and feces respectively, of one subject, 1 with organisms from the throat of another, and the fourth with fecal streptococci from a third subject. The work here reported was done with 4 biologically distinct serums.

¹¹ Brit. Med. Jour., 1921, 1, p. 632.

Agglutination tests were made with the serums against my series of 46 cultures. These streptococci create some difficulty by their tendency to agglutinate spontaneously. This was entirely eliminated by the use of the dextrin medium previously described by me.¹² The results of the agglutination tests were not entirely consistent. Certain strains were agglutinated by more than one serum, although homologous strains for the serums did not interagglutinate. The complete data are not given here as they serve only to confirm previous observations to the effect that this group of organisms tends to produce group agglutinins, that some strains are capable of being agglutinated by a number of serums, and finally that agglutination tests are insufficient to establish biologic identity of the organisms in this group. The discrepancies in the agglutination tests were eliminated when absorption experiments were made.

For the absorption tests a method was followed similar to that used by Gordon¹¹ for grouping hemolytic streptococci: 100 c c of dextrose broth to which had been added a few drops of ascites fluid was inoculated from a 24-hour blood-agar slant of the strain to be tested. After 24 hours' incubation the suspension was divided into 2 portions and both centrifuged for 30 minutes. The supernatant liquid was then drawn off and 1.5 c c of diluted (1:100) immune serum added to one portion. After thoroughly shaking, the mixture was incubated at 37 C. for 1 hour and centrifuged. The partially absorbed serum was then added to the other portion of centrifuged organisms, incubated and again centrifuged. The supernatant liquid from this was then tested against a suspension of the organisms homologous for the serum to determine the presence or absence of agglutinins. Only complete absorption of agglutinin was taken as significant. When partial absorption took place the test was repeated with a larger amount of culture.

Four biologically distinct serums were tested with 46 strains of *Streptococcus viridans*. The strains which gave complete absorption are recorded in the following table.

TABLE 1
ABSORPTION OF AGGLUTININ

Serum	Strains Which Absorbed Agglutinin
1	A5, A6, A9, B2,* B5, B6, BF6, CF3, D8, D10
2	AF12,* AF13, BF8
3	AF11, C1, C4, DF1,* DF2, DF4
4	CF11, GF6, GF8*

An asterisk denotes the homologous strain. The first letter designates the subject and F as the second letter indicates a fecal strain. Thus A5 is a throat strain from subject A and CF11 a fecal strain from subject C.

¹² Jour. Am. Med. Assn., 1921, 76, p. 1753.

The agglutinin in serum 1 was absorbed by 10 strains. These included 3 throat strains from each of 2 subjects, a fecal strain from 1 of them, 2 throat strains from a third person and 1 fecal strain from a fourth. Serum 2 reacted with 2 fecal strains from 1 subject and 1 from another. Serum 3 reacted with 2 throat strains and 4 fecal strains from 2 persons, while the agglutinin in serum 4 was absorbed by 3 fecal strains from 2 subjects.

In all cases other strains both from the throat and the feces failed to absorb, although the strains were picked from the same plate as those which did absorb. Strains AF 11 and AF 12 absorbed different serums, although from the same plate, as did also CF 3 and CF 11.

The results here reported establish certain facts. The non-pathogenic green-producing streptococci are biologically heterogeneous. There is no correlation between cultural characters and biologic reactions or between source (i. e., throat or intestines) and agglutinin absorbing power. A person harbors at the same time both in the throat and in the intestinal tract streptococci that are biologically distinct. Jordan and Sharp¹³ have recently reported similar findings for the group of Pfeiffer bacilli found in the throat and suggest that definite biologic properties are associated only with definite disease-producing power. Groups of biologically identical streptococci associated with a disease process have been reported by Rosenow⁸ from cases of influenza on the basis of the agglutination reaction, by Tunnicliff⁷ from influenza using the opsonic technic, by Herrold⁹ from chronic prostatitis on the basis of agglutination and absorption tests and by others. Apparently we cannot expect to obtain a simple biologic classification of the whole *Streptococcus viridans* group, both pathogenic and nonpathogenic varieties.

SUMMARY

The agglutinin absorption reaction is more reliable than the more simple agglutination test as a means of identifying organisms of the *Streptococcus viridans* group.

There is no correlation between cultural and biologic groupings.

One person may harbor at one time more than one strain of *Streptococcus viridans*.

The green-producing streptococci (*Streptococcus viridans*) comprise a group of organisms biologically heterogeneous, the agglutinin absorption test being used as a criterion, and no simple classification is possible unless definite pathogenic properties develop.

¹³ Jour. Infect. Dis., 1922, 31, p. 198.

THE PRODUCTION OF SPASMS OF THE DIAPHRAGM IN ANIMALS WITH A STREPTOCOCCUS FROM EPIDEMIC HICCUP

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In a preliminary report,¹ attention was directed to the isolation of a streptococcus from 3 cases of epidemic hiccup, with which spasms of the diaphragm were reproduced in animals. The close relationship of this condition to epidemic lethargic encephalitis, as suggested by clinical and epidemiologic observations, was discussed, and experimental results in support of this view were reported. Thus far, I have found only one reference in the literature on experimental research in epidemic hiccup, and that is the one by Pontano and Trenti,² who inoculated volunteers with blood, spinal fluid, and throat washings obtained during the febrile period of epidemic singultus. The results were negative.

In this paper I shall record details of an experimental study of 8 cases of epidemic hiccup.

TECHNIC

In this as in similar studies, painstaking effort was made to procure material from the depths of a given focus, contaminated as little as possible with secretions from the mouth. Patients were asked to rinse their mouths with water. Swabs from the nasopharynx were taken without touching the tongue, by means of cotton wrapped on the end of a curved wire. Suspensions of pus expressed from pockets in tonsils and aspirated from pyorrheal pockets, and suspensions of the swabs were made according to routine in 2 cc of salt solution. From this, horse blood-agar plate and glucose-brain-broth cultures were prepared.

The cultures were incubated in a moist atmosphere at from 33 to 35 C. Two rabbits were injected intracerebrally under ether anesthesia with the suspensions in salt solution, one usually received 0.1 to 0.2 cc, and one 0.2 cc of a 1:10 to 1:1000 dilution, depending on the density. At first, suspected colonies on blood-agar plates were grown in glucose-brain broth and injected, but since characteristic symptoms were not obtained the procedure was dropped. The primary glucose-brain-broth culture was used for routine injections after abundant growth had occurred, or after inoculation for from 18 to 24 hours. In an attempt to maintain specific infecting powers, the cultures in glucose-brain broth were made by inoculating approximately 0.2 cc of each preceding culture into approximately 20 cc of warmed broth; the culture was transferred as soon as growth occurred, from 4 to 6 times a day, and the night cultures were not allowed to become more than 10 hours

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¹ Rosenow, E. C.: Jour. Am. Med. Assn., 1921, 76, p. 1745.

² Policlinico, 1921, 28, p. 1163.

old. Usually, 0.2 cc of the undiluted culture and 0.2 cc of a 1:100 or 1:1000 dilution were injected intracerebrally under ether anesthesia into rabbits and monkeys, and from 0.2 to 5 cc intravenously into rabbits, depending on the circumstances and the purpose of the experiment. Before inoculation, the animals were anesthetized and the hair was clipped and thoroughly disinfected with an alcohol sponge. An awl $\frac{1}{8}$ inch in length, sterilized in the flame, was used as a trephine, and inoculation into the right frontal lobe was made through the skull, a little to the right of the median line at the level of the posterior angle of the eyes. Care was exercised to make the injections as nearly alike as possible, an attempt being made to inject some of the material into the right lateral ventricle. Loss of fluid after withdrawal of the needle was prevented by immediately applying firm pressure for a short time with sterile gauze. The procedure was quite harmless and readily accomplished. The rabbits were given oats, hay, greens and water. They were observed at stated intervals morning, noon, and night, and in special instances during most of each day and night, and the symptoms were recorded as they developed. At first some difficulty was experienced in differentiating spasms of the diaphragm from spasms of the abdominal muscles; but it was soon found that in the former the abdominal walls were suddenly thrown outward as the abdominal viscera were forced backward, due to contractions of the diaphragm, an occurrence which did not take place in spasms of abdominal muscles. The contractions in the abdominal muscles could readily be palpated. The diaphragmatic contractions were easily recorded on a revolving drum of smoked paper; a band around the animal's abdomen was connected to a tambour and recording lever.

Necropsies were made as soon after death as possible. The cultures of the brain and the spinal fluid were made by opening the skull in a sterile manner, turning back the vertex, and pipetting the substance of the brain, and the ventricular and spinal fluids, and inoculating blood-agar plates and glucose-brain broth. Cultures of the blood from the heart, 0.2 to 0.5 cc, were also always made in the same mediums. The brain, medulla and cervical cord were examined and placed in 10% formalin for microscopic study.

Control experiments were undertaken with filtrates prepared by passing thoroughly emulsified, nasopharyngeal washings and brains of rabbits that developed spasms of the diaphragm, and cultures from these, through bacterial filters of the Mandler type. These were controlled with *B. prodigiosus*. Cultures were made in glucose-brain broth, meat infusion, and ascites tissue fluid layered with liquid petrolatum. Injections of from 1.5 to 2 cc were made intracerebrally in the usual way, depending on the size of the rabbits.

Blocks for microscopic study were taken according to routine from the cerebral hemisphere, the pons, the medulla, including the posterior part of the cerebellum, and the cervical cord. The sections were stained for lesions with hematoxylin and eosin, and for bacteria by the Gram method, but methyl violet instead of gentian violet was used. The sections were decolorized to a pale blue, so that the nuclei of cells were pale blue and the bacteria dark purple, almost black in transmitted light.

DETAILS OF EIGHT CASES OF EPIDEMIC HICCUP

CASE 1.—Mr. R. M., an epileptic in the Rochester State Hospital, developed hiccup during the night of Feb. 20, 1921. This persisted almost continuously until Feb. 22, when cultures were made; the hiccups recurred at long intervals for another day, and then disappeared. The abdominal walls were thrown suddenly outward with each spasm of the diaphragm, when the air was

drawn audibly through the spasmodically narrowed orifice in the glottis. The spasms varied greatly in intensity at irregular intervals, some being slight and not distressing, while others were severe and associated with pain along the lower costal border. During the height of an attack the patient became exhausted. His epileptic seizures occurred unchanged during and after the attack. Examinations showed normal temperature, urine, blood culture, and teeth. There were no spasms of other muscles; no vomiting or distress was present after meals. The leukocytes numbered 14,000. The tonsils were large, the crypts deep, and the nasopharynx was moist and hyperemic.

At examination, a small abscess in the pole of the left tonsil was ruptured, and a small amount of this pus and washings from the nasopharyngeal swab were suspended in salt solution, grown, and injected intracerebrally into one rabbit each; the pus from the tonsil was injected intravenously into one rabbit. Cultures from the tonsil pus, and throat swab yielded countless numbers of green-producing streptococci, a few hemolytic streptococci, staphylococci, and *Micrococcus catarrhalis*.

Results of Animal Experiments in Case 1.—The rabbit injected intravenously with pus from the tonsil remained well. The animal injected with the suspension from the nasopharynx had slight spasms of the diaphragm for a day, and then recovered. The rabbit injected intracerebrally with 0.2 cc of the suspension of the pus from the tonsil developed marked rhythmic spasms of the diaphragm on the fifth day. The details in this experiment (rabbit 2283) have been reported.¹

An emulsion of the brain of this rabbit was injected intravenously into 1 rabbit and intracerebrally into 2 rabbits and 1 monkey (second animal passage). The rabbit injected intravenously remained well. The 2 rabbits injected intracerebrally developed a remarkable train of symptoms. Both sat around quietly the day after injection, often with eyes half closed, responding slowly to stimuli. On the second day the respirations became increased and jerky. Tremor and twitching of the muscles of the anterior part of the body developed in both. One developed rhythmic spasms of the diaphragm, usually synchronous with lesser spasms of the muscles of the fore and hind extremities, and it ran about in the cage in an incoherent manner. Both died on the third day after injection, apparently from respiratory failure, without convulsions. The monkey, injected with 0.3 cc, developed clonic spasms of the muscles of the head and neck, and had repeated generalized convulsions.

Both rabbits had marked congestion of the vessels of the meninges, punctate hemorrhages in the pia over the anterior portion of the medulla, and distinctly turbid cerebrospinal fluid. Hemorrhages were not found in the phrenic or vagus nerves or in the diaphragm. Large numbers of green-producing streptococci in pure culture were isolated from the brain, spinal fluid, and blood of both rabbits. The monkey died in 36 hours. Necropsy revealed mild meningitis. Countless numbers of streptococci were found in the spinal fluid and blood.

Of the brain emulsion of the rabbit that had spasms of the diaphragm 0.5 cc was immediately injected intravenously into one rabbit, and 0.1 cc intracerebrally into another (third animal passage). Both animals became sleepy, developed tremor and increased tonus of the muscles of the neck and fore extremities, and had marked weakness and clonic spasms of the hind extremities. Death occurred on the second and third days, respectively.

The necropsy findings were similar in both animals. The cerebrospinal fluid was distinctly turbid; the vessels of the meninges were congested, and

there was a moderate number of hemorrhages in the pia on the under surface of the cerebrum, pons and medulla. Besides these lesions, hemorrhages throughout the length of the cord were found in the rabbit that was injected intravenously.

The brain emulsion of the rabbit injected intravenously was then injected intracerebrally into one rabbit, and the brain emulsion of the rabbit injected intracerebrally was injected into one dog (fourth animal passage). The rabbit had markedly increased respiration the day after injection; it was shaky, tremulous, hyperesthetic, and had occasional twitchings of the muscles of the right ear. It grew rapidly worse and died 36 hours after injection. At necropsy, marked meningitis was found, with countless numbers of the streptococcus in brain emulsion, spinal fluid, and blood.

The day after injection the dog was nervous, salivated, yawned frequently, and the latter part of the day developed explosive cough, but it had no spasms of muscles and was quite playful. On the third day, the animal was not so playful and rhythmic clonic spasms of the muscles of the neck and fore extremities had developed, averaging 60 to 70 a minute, and at intervals there were synchronous twitchings of the muscles of the ears. The animal often changed its position, especially after the more violent spasms, including those of the diaphragm, in an apparent effort to control them. The following day the spasms of the fore extremities continued, and outspoken rhythmic spasms of the diaphragm developed. The animal was found dead on the morning of the fourth day.

Necropsy revealed a large hemorrhage over the entire right hemisphere, and purulent exudate surrounding the medulla and cervical cord, but no gross lesions in the brain or cord substance. Cultures and smears from the spinal fluid yielded countless numbers of streptococci.

The primary culture (second animal passage) of the brain of rabbit 2283 was injected intracerebrally into one rabbit. It was shaky in the fore extremities the morning after injection, developed marked weakness during the day, but had no spasms of the diaphragm. The next morning it was found dead. Necropsy revealed a moderate number of hemorrhages throughout the cord substance, most marked in the cervical region. There were no hemorrhages in the brain.

One-tenth of a cc of the primary glucose-brain-broth culture from the brain of this rabbit (third animal passage), which yielded a pure culture of streptococcus, was injected intracerebrally into one rabbit. The animal died on the third day of meningitis without developing spasms of the diaphragm.

The primary culture of the tonsil pus in glucose-brain broth, which on plating yielded an almost pure culture of the green-producing streptococcus, was injected intravenously into 2 rabbits, 1 of which remained well. The findings in the other were as follows:

Rabbit 2286, weighing 1,500 gm., was injected intravenously Feb. 23, 1921, with 3 cc of the primary glucose-brain-broth culture of the tonsil pus. The animal remained well until March 2, when it sat around quietly, hopped slowly, and the eyelids drooped. March 3, its condition was about the same, but it appeared rather uncomfortable, disinclined to move, and when it was prodded, undoubted spasms of the diaphragm were noted, but no spasms of muscles elsewhere. The following 2 days the animal was very quiet, and distinctly slow; it held its head in extended position, often resting it over the shoulders of its mates, and its eyes were half closed, but there were no further spasms of the diaphragm. March 6, it was found dead.

Blood-agar-plate cultures of the brain and blood were sterile. Glucose-brain-broth culture of the blood was also sterile, while the cultures from

the brain yielded short chained diplostreptococci. The sections revealed a variable degree of round-cell infiltration around a group of blood vessels in the pons and medulla (fig. 1), and in circumscribed areas on the inner aspect of the left lateral ventricle. There was no infiltration around the ependymal cells in the choroid plexus, but the vessels were dilated. A few diplococci were demonstrable in the areas of round-cell infiltration adjacent to the lateral ventricle, and in one vessel which showed slight perivascular infiltration there was a mural hyalin thrombosis. Two leukocytes and one endothelial cell were found which contained a number of undoubted small gram-positive diplococci. One leukocyte containing the diplococci was apparently migrating through the wall of the vessel.

The possibility that the streptococcus isolated in pure culture from these animals was a secondary invader to some ultramicroscopic organism was considered. The strain isolated from the brain of rabbit 2283 was rapidly transferred successively into warmed glucose-brain broth as soon as growth

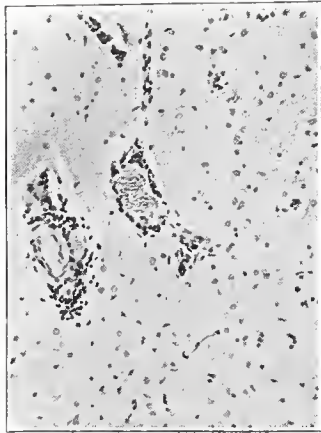


Fig. 1.—Perivascular round-cell infiltration in the pons of rabbit 2286 injected intravenously. Hematoxylin and eosin; $\times 100$.

occurred, and animals were injected at intervals with these cultures as well as with filtrates of the brain emulsions and cultures of animals with spasms of the diaphragm. In the seventh culture generation this streptococcus produced spasms of the diaphragm in 1 of 4 guinea-pigs injected intratracheally. In the thirty-third subculture the organism was injected intracerebrally into a rabbit, a monkey, and a dog. The rabbit developed rhythmic spasms of the eyelids and of the muscles of the left side of the abdomen. The monkey developed spasms of the diaphragm. Both animals became lethargic for a time and then recovered. The dog, however, died, apparently of meningitis. Of the filtrate from the brain of the rabbit, which yielded a pure culture of streptococcus in glucose-brain broth, 1.5 cc were injected into the brain of 1 rabbit and 1 monkey. The monkey died in 48 hours from streptococcal meningitis. The rabbit remained well for 3 days, and then developed tremors of the muscles of the back, with occasional sharp clonic spasms, and rapid, labored respirations. On the fourth day it had continuous rhythmic spasms of the diaphragm, associated with hiccup in the more severe spasms, and shortly before death it developed retraction of the head.

Necropsy revealed turbid spinal fluid, subdural hemorrhage opposite the roots of the second, third, and fourth cervical nerves, edema and infiltration of the pia over the anterior aspect of the medulla and pons, and countless streptococci in the brain.

In the thirty-seventh subculture in glucose-brain broth this strain was injected intracerebrally into 4 rabbits and 4 monkeys in doses varying from 0.5 cc of the undiluted culture to 0.1 cc of a 1:1000 dilution of an actively growing 3-hour culture. It was also used to infect the traumatized pulps of the teeth of 1 rabbit. Three of the rabbits and 3 of the monkeys injected intracerebrally developed persistent spasms of the diaphragm. Necropsy of the fourth rabbit revealed a large hemorrhage in the right frontal lobe at the point of injection. The fourth monkey became comatose without developing spasms.

Rabbit 2338, weighing 1720 gm., was injected intracerebrally March 8, 1921, with 0.1 cc of 1:100 dilution of a 3-hour glucose-brain-broth culture in the thirty-seventh culture generation. The animal appeared well until March 11, when it acted peculiarly, hopped around as if weak in the hind extremities, and had occasional spasms of the muscles of the back, especially after hopping. March 12, at 7:30 a. m., it sat quietly in the cage with the hind quarters flat, but stood quite erect on the fore extremities and

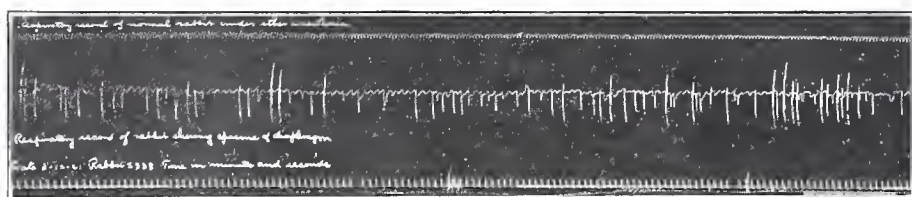


Fig. 2.—Respiratory record of rabbit 2338 showing spasms of the diaphragm following intracerebral injection of the streptococcus in the thirty-seventh subculture.

breathed rapidly as continued clonic spasms of the diaphragm occurred with occasional distinct hiccups. At 9:30 a. m., the spasms of the diaphragm had disappeared and the animal had developed continuous rhythmic spasms of the muscles of the neck with sharp turning of the head to the right, horizontal nystagmus, spasms of the muscles of the shoulder, and almost complete paralysis of the hind extremities. At 2 p. m., the animal lay on its side and occasionally had sharp tremor and twitching of the muscles of the fore extremities; this occurred usually synchronously with continuous although irregular spasms of the diaphragm which varied greatly in intensity (fig. 2). At 5 p. m., the spasms of the diaphragm had become more irregular in time and degree as the animal lay quietly on its side in a relaxed condition, breathing normally. At 10 p. m., the spasms of the diaphragm became rhythmic. March 13, from 7:30 a. m., to 10 a. m., the animal was relaxed and lay quietly on its side; respirations were normal, and there were no spasms of muscles. Its head was held in retracted position, but could easily be flexed; this provoked tonic contractions of the muscles of the extremities following which the animal again became relaxed. The animal was chloroformed.

Necropsy revealed an exudate between the cerebellum and the cerebrum, subdural hemorrhage in the cervical region of the cord, and large areas of hemorrhage and edema in the subdural space opposite the roots of the fifth

cervical nerve. The pia covering the pons and medulla was edematous. The anterior lobes of the hypophysis were edematous and hyperemic, while the posterior lobes appeared normal. There was no evidence of diffuse meningitis; there were no lesions in the substance of the lumbar cord.

Cultures from the brain and cerebrospinal fluid yielded large numbers of the characteristic streptococcus, whereas, those from the blood were sterile. Microscopic examination revealed a large number of round cells and leukocytes in the pia over the anterior aspect of the medulla and pons, in the perivascular spaces in the pons and medulla, and in areas of infiltration in the anterior nerve roots (fig. 3).

Rabbit 2357, weighing 1100 gm., was injected intracerebrally March 13, 1921, with 0.2 cc of salt solution. The pulp chambers of the 4 incisors were then drilled into and the pulp severely traumatized and infected with a small amount of the emulsion of the brain of rabbit 2338; the openings were sealed with dental cement. March 14, the animal appeared well. March 15, it disinclined to hop, sat quietly, and crouched flat to the floor of the cage repeatedly, apparently in an attempt to control spasms of the diaphragm which occurred rhythmically for several hours. March 16, the animal was found dead.

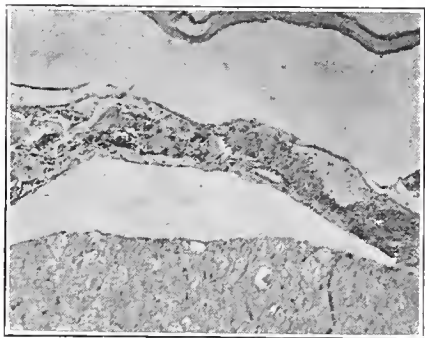


Fig. 3.—Edema, hemorrhage, and leukocytic and round-cell infiltration of the anterior cervical nerve root of rabbit 2338. Hematoxylin and eosin; $\times 90$.

Necropsy revealed moderate edema and infiltration in the lower jaw opposite the roots of the lower incisors, turbid cerebrospinal fluid, marked congestion of the vessels of the meninges, and hemorrhages opposite the roots of several cervical nerves.

Cultures from the brain, cerebrospinal fluid, blood, and pulp canals of the infected teeth yielded countless numbers of the characteristic streptococcus. Sections of the brain and cord showed no areas of perivascular, round-cell infiltration in their substance, but there were edema and round-cell infiltration of the pia over the medulla and cervical cord, especially around the blood vessels adjacent to cervical nerve roots and in the anterior fissure. In both locations gram-positive diplococci were demonstrated.

March 8, 1921, monkey 254 (*Maccacus rhesus*), weighing 2.5 kg., was injected intracerebrally with 0.2 cc of a glucose-brain-broth culture of this strain in the thirty-seventh subculture. March 9, at 11:45 a. m. the animal appeared sick. It had difficulty in breathing, respirations being short and jerky, and at intervals it had audible hiccup, due to spasms of the diaphragm and glottis. The extremities were weak, and the left side of the face was drawn; it was extremely hyperesthetic. When its back was stroked clonic spasms devel-

oped in most of the muscles of the body, including the diaphragm. At 3:30 p. m. the picture had changed markedly. The animal lay relaxed in the cage, and had developed rhythmic, continuous, marked hiccup, a rather loud inspiratory sound occurring with each spasm of the diaphragm. This continued for half an hour, when a respiratory tracing was taken (fig. 4a). Soon after, and while the tambour was still in place, the diaphragm began to fibrillate as the animal developed great difficulty in breathing. The contractions became gradually more feeble, but continued at a rapid rate until death (fig. 4b).

Necropsy revealed mild meningitis, hemorrhagic areas over the lower rolandic area and the pia on the right side, and turbid cerebrospinal fluid. There were no gross hemorrhages in the cord, and no lesions of the phrenic nerve or diaphragm.

Cultures from the blood and spinal fluid yielded the characteristic streptococcus in pure culture. Sections of the brain and cord revealed leukocytic infiltration of the meninges, and perivascular infiltration by leukocytes and

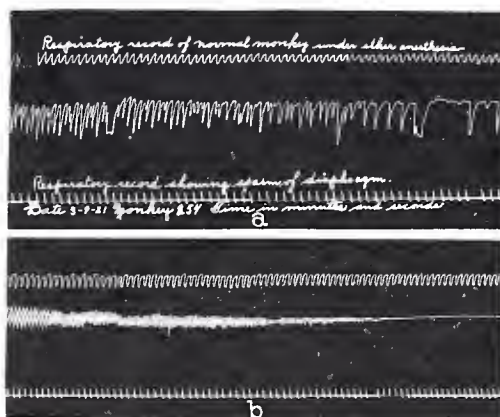


Fig. 4.—*a*, Respiratory record of monkey 254 injected intracerebrally with the streptococcus in the thirty-seventh subculture showing spasms of the diaphragm; *b*, fibrillation of diaphragm as animal died.

round cells extending deep into the subcortical region of the cerebrum (fig. 5), in which the streptococcus was demonstrable.

Rabbit 2359, weighing 2000 gm., was injected intracerebrally March 13, 1921, with 2 cc of the filtrate of a mixture of the brain emulsions of the 3 rabbits that developed spasms of the diaphragm. This had yielded a pure culture of the streptococcus in glucosc-brain broth. The rabbit was well on March 14, 15 and 16, but on the seventeenth, it disinclined to move, and when made to do so, it responded slowly. The following day its movements were even slower. March 19, it remained quiet, half asleep most of the time, and responded slowly to stimuli. March 20, this condition had become more marked. It paid no attention to its surroundings or to mild prodding. March 21, it continued in this sleepy condition, did not respond to slight stimuli, and was slow in responding to rather severe prodding. It resisted only slightly when placed on its side or back, and continued in apparent sound sleep in these positions. Fine tremor of the right fore leg was noted, and occasional sharp twitching of the muscles. Holding a carrot to its mouth was not suf-

ficient to arouse it, but when the carrot was placed in its mouth it appeared to waken, masticated for a short time, apparently with relish, and then seemed to forget to swallow. March 22, it was found dead.

Necropsy failed to reveal gross lesions of the brain or the cord, but cultures yielded a small number of the characteristic streptococcus. Sections showed moderate perivascular infiltration in the pons, but none in the cerebral cortex and medulla.

The filtrate of a mixture of the emulsion of the brain of 2 monkeys that had hiccup yielded characteristic streptococcus in cultures. This was injected intracerebrally into 1 rabbit and 1 dog. Gauze soaked in the emulsion was used to pack the nose of 1 monkey, and the primary culture in glucose-brain broth from the brain in dense suspension was inoculated into the teeth of 1 dog. The rabbit became extremely lethargic, but had almost entirely recovered 12 days after injection, when it was chloroformed. The details of the findings in this animal have been published.¹ The dog injected with 3 c c

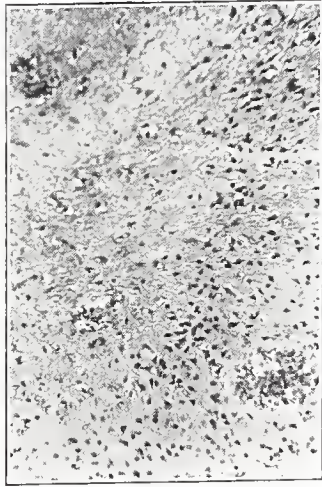


Fig. 5.—Perivascular infiltration, and focal necrosis in the subcortical region of monkey 254. Hematoxylin and eosin; $\times 100$.

of the filtrate was quite well the following day, but on the second day developed undoubted spasms of the diaphragm, which occurred synchronously with from every second to fifth inspiration. The monkey developed a mucopurulent discharge from the nostrils, became hoarse, and had a cough; it was slow in action and in response to stimuli, but did not develop spasms. It died on the sixth day. Necropsy revealed tracheobronchitis and bronchopneumonia, but no gross or microscopic lesions of the brain.

The dog whose 4 cuspids were devitalized and infected with a dense suspension of the organism appeared nervous the day after injection, shivered almost constantly, licked its jaws frequently, and had a number of spasms of the diaphragm. On the second day the animal developed repeated sharp spasms of the diaphragm. On the fourth day it had recovered. Swelling of jaws or gums around the infected teeth was not observed, and the teeth remained firm in their sockets.

The seventy-third subculture was injected into 2 rabbits. Both animals developed a very similar train of symptoms. They became quiet and slow.

On the second day, they appeared to be half asleep, and there were sharp twitchings of the masseter muscles, tremor and clonic spasms of the muscles of the fore extremities and shoulders, no spasms of the diaphragm, markedly increased respirations, and marked weakness of the extremities. Both rabbits died within 48 hours.

The spinal fluid was markedly turbid; there were no lesions at the point of inoculation. One animal had a number of hemorrhages in the choroid plexus; in the other there were no gross hemorrhages. Cultures from the blood, and brain emulsion of both yielded large numbers of the organism injected.

The filtrates with which positive results were obtained in the above experiments yielded a pure culture of the characteristic streptococcus in glucose-brain broth. Seven additional filtrates, but which proved sterile, were made and injected intracerebrally into 10 rabbits. All remained free from symptoms and lesions.

Of the 26 animals injected intracerebrally with suspensions of the bacteria from the throat and tonsils, with cultures of the characteristic streptococcus, and with filtrates proved to contain the organism by cultural methods in from 1 to 4 animal passages, 13 had spasms of the diaphragm, 10 became lethargic, and 7 developed suppurative meningitis. The 2 rabbits injected in the first animal passage developed spasms of the diaphragm without lethargic symptoms or meningitis. Fifteen animals were injected with this strain in the second animal passage, some after many subcultures, one subculture being the seventy-third. Nine developed spasms of the diaphragm, 5 became lethargic, and 2 developed meningitis. Of the 8 injected in the third animal passage, only 1 developed spasms of the diaphragm, 5 became lethargic, and 3 developed suppurative meningitis. Of the 2 injected in the fourth animal passage, 1 developed spasms of the diaphragm, and necropsy revealed meningitis in both.

Spasms of the diaphragm followed intravenous injection of the organism in one (rabbit 2286) of 3 rabbits, and in 1 whose teeth were infected immediately after injecting sterile salt solution intracerebrally (rabbit 2357). One of 4 guinea-pigs inoculated intratracheally with 0.5 cc for each 100 gm. of body weight of the strain in the second animal passage in the seventh subculture, developed spasms of the diaphragm. Cultures of the blood after death were negative; those from the brain emulsion yielded a pure growth of the streptococcus.

A summary of the symptoms in the 26 animals (19 rabbits, 3 dogs, and 4 monkeys) is given in table 1.

Cultures of the blood and brain were made after death in 29 animals which were injected intracerebrally or intravenously, or in which the teeth or nasal tracts were infected with the cultures. The cultures were sterile in 4, none of which had symptoms during life; the duration of the experiments was 6, 9, 11, and 12 days, respectively. Two of the animals were injected with sterile filtrates, 1 intravenously with the primary culture from the tonsil pus; the nose of the other was packed with gauze soaked in the culture.

The characteristic streptococcus was isolated in pure culture from the brain in the remaining 25, and from the blood in 18, in from 1 to 6 days after inoculation.

Intraperitoneal injections into mice of pure cultures of the streptococcus showed it to be of a low grade of virulency.

March 4, nine days after the patient's attack had ceased, the infecting power of the bacteria from the tonsils and throat was again studied; emulsions of pus expressed from the tonsils, and suspensions of the nasopharyngeal swab

TABLE 1
INCIDENCE OF VARIOUS SYMPTOMS IN ANIMALS FOLLOWING INOCULATION OF THE STREPTOCOCCUS FROM EPIDEMIC HICCUP

Cases	Strains	Duration of Symptoms, Days	Number of Animals Studied	Muscular Spasms			Abnormal Movements and Posture					Tremor	Restlessness	Hyperperneation	Convulsions	Paralysis	Lethargy
				Diaphragm	Abdominal	Other	Total	Nystagmus	Rhythmic Movements	Tic-like Movements	Ataxia	Turning of Head	Retraction of Head				
4176 (1)		3	26	13	5	10	19	2	4	0	5	1	6	9	1	2	10
4204 (2)		42	9	5	0	6	8	2	2	1	4	0	0	8	2	0	3
4281 (3)		4	9	4	0	6	8	3	2	2	4	4	2	7	2	5	3
4573 (4)		90	17	7	4	7	12	4	4	3	8	3	2	10	1	1	1
4589 (5)		2	16	1	1	6	8	5	3	2	4	1	1	3	5	2	0
4596 (6)		7	4	3	0	1	4	0	0	1	2	1	2	3	3	3	2
4602 (7)		3	9	5	5	8	8	3	1	1	6	5	2	8	5	5	1
4621 (8)		3	6	2	1	4	4	2	0	0	3	3	0	3	2	1	1
Total.....			96	40	16	48	71	21	16	10	36	18	15	51	21	6	21
First and second animal passages.....			45	26	10	33	39	13	5	7	22	15	7	30	17	5	13
After two or more animal passages and after many rapidly made subcultures.....			51	14	6	15	32	8	11	3	14	3	8	21	4	1	17

were each injected intracerebrally into 1 rabbit. The rabbit injected with the tonsil pus developed marked tremor of the muscles of the fore extremities and marked weakness, but did not have spasms of the diaphragm. The one injected with the suspension of the nasopharyngeal swab remained well for 8 days, when it had repeated clonic spasms of the diaphragm for 1 day, and then recovered.

CASE 2.—Mr. O. J. L., aged 76 years, entered the Clinic April 4, 1921, complaining of stomach trouble, and a persistent hiccup of 6 weeks' duration. About 3 days prior to the beginning of the hiccup, the patient was taken ill with "cold" in the head, fever, chill, general aching, and malaise, but did not go to bed. The rest of the family had, at about the same time, what was considered grip; none developed hiccup, but in the same community, one of the patient's friends, with whom he had not come in direct contact, had a similar attack. The hiccup was very severe and continued almost constantly for 10 days, when it disappeared for about a week. The attack returned, however, and the hiccup grew more frequent each day until it became more or less constant night and day. It had continued paroxysmally ever since, and kept the patient awake and sitting up most of the night. He was clear mentally and did not have headache or diplopia. The hiccup exhausted him, and when it gave what he termed the double stroke, he experienced much pain in the lower region of the chest. Since the attack he had been extremely constipated so that 6 to 7 days sometimes passed without his having a bowel movement. At these times he was dizzy, bloated, and nauseated.

On April 4, the blood pressure was 170 systolic and 95 diastolic; the pulse was 70, the temperature normal, and the urine negative. Diagnosis was made of moderate arteriosclerosis, and a small epithelioma of the inner canthus of the right eye. Neurologic examination was negative. Examination of the mouth showed many snags and roots of teeth, and red and swollen gums. The tonsils were small, but pus was expressed from the upper poles.

The results of animal experiments in this case were similar to those in Case 1. Of the 9 animals injected intracerebrally with suspensions in salt solution of the tonsil pus, and nasopharyngeal washings, with respective primary cultures, and with subcultures of the characteristic streptococcus after 1 and 2 animal passages, including the forty-fifth rapidly made subculture, 5 developed spasms of the diaphragm of varying intensity, and 3 became lethargic (table 1). Cultures made from the brains of animals that succumbed in from 1 to 3 days after inoculation yielded the characteristic streptococcus in all but 1; the blood was sterile in all. Symptoms did not develop, cultures were sterile, and no microscopic changes were found in the central nervous system in 7 rabbits and 1 monkey injected intracerebrally, respectively, with the filtrate of the nasopharyngeal washings, spinal fluid, and the sediment of the dehemoglobinated blood of the patient.

This strain was agglutinated specifically by anti-encephalitis serum. Protection experiments were, therefore, undertaken in mice inoculated in series. Of the serum, 0.2 cc was injected subcutaneously at the time of intraperitoneal injection of the streptococcus. It protected completely against 0.5 cc which killed control mice within 48 hours, as well as the additional controls injected with normal horse serum.

CASE 3.—Mr. F. H., aged 42 years, came to the Clinic April 15, 1921, seeking relief from persistent hiccup. The patient had had grip during January and February and had been confined to his bed most of February and March. The hiccup which began in the morning 3 days before he came to the Clinic, had interfered with his work. The morning he came to the Clinic,

it had ceased for 2 or 3 hours. He felt like belching, but when he tried to raise the gas the hiccup commenced. Any temporary excitement tended to stop it for a few moments, but it always started again when he was quiet and when he smoked. During the 4 days of observation, the hiccup continued in spite of the use of bromides. The spasms of the diaphragm were severe, occurred irregularly, synchronous with from the second to the sixth inspiration. The respirations were irregularly timed and of varying amplitude (fig. 6). The patient believed the bromides made him worse. Benzyl benzoate relieved him somewhat. He fell unconscious one night on the floor of his room. Finally, on the fourth day after his admission, the hiccup stopped suddenly and did not return.

On account of the hiccup, examination of the abdomen was difficult, but it was found to be slightly tender on both sides and in the epigastrium. The fingers and hands were decidedly tremulous. The blood pressure, the pulse rate, and the urine were normal. The hemoglobin was 80%, the leukocytes numbered 7,800, and the Wassermann test was negative. The tonsils were large and moderately infected, and the pharynx was red and moist. Many of the teeth had been crowned; there was marked pyorrhea; roentgen-ray examination revealed apical abscesses over 6 teeth.

The material expressed from the tonsils and the pus aspirated from the pyorrheal pockets were each suspended in 2 cc of salt solution, cultures were made, and 2 rabbits were inoculated intracerebrally with each suspension. The

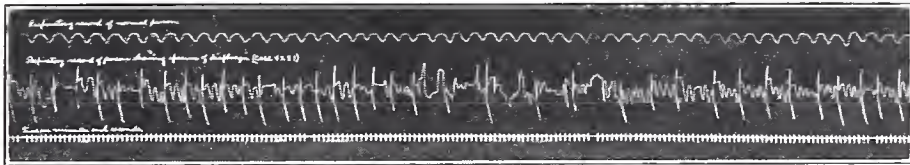


Fig. 6.—Respiratory record of case 3, showing spasms of the diaphragm and irregular respirations.

cultures yielded a preponderance of colonies of the green-producing streptococcus. The 2 rabbits inoculated intracerebrally with the pyorrheal pus remained free from symptoms.

Results of Animal Experiments in Case 3.—Rabbit 2541, weighing 1830 gm., was injected intracerebrally, April 16, 1921, with 0.2 cc of the salt solution suspension of the tonsil pus. April 17, the animal appeared well. April 18, at 7 a. m. it disinclined to move. At 12 m., the respirations were decidedly increased; the animal was hyperesthetic, tremulous and ataxic, and slight lateral oscillations of the head were noted. At 8 p. m. the lateral movements of the head had become more marked; ataxia was more severe; horizontal nystagmus, usually synchronous with respirations, had developed. There were no spasms of the muscles. A respiratory tracing was taken (fig. 7). At 10 p. m., the animal sat quietly in the cage and seemed afraid to move. When made to do so, ataxia and marked tremor of the fore part of the body were noted. The nystagmus and lateral movement of the head continued, and the animal occasionally turned its head sharply to the left or right and under the fore part of the body. April 19, at 1 a. m., the condition was about the same, but the lateral movement of the head was less marked. There were no spasms of muscles. At 4 a. m., the animal seemed uncomfortable, moving carefully. It had developed occasional sharp spasms of the diaphragm. At

6 a. m., a regular, rather frequently repeated sound resembling hiccup was heard; on observation, this was found to be associated with violent, sharp, frequently repeated spasms of the diaphragm. The animal seemed afraid to move. At 7 a. m., the condition was unchanged. At 8:45 a. m., the animal appeared uncomfortable as spasms of the diaphragm, of varying intensity, continued. The lateral movements of the head and the nystagmus had ceased. The animal seemed thirsty and began to drink water, but as it swallowed, it became excited, as if swallowing were painful, and afterward drew its head away when water was brought to its mouth. Sometimes the spasms would cease for a few minutes and then recur. At 9:25 a. m., rapidly repeated spasms of the diaphragm of varying intensity occurred (fig. 7). These continued with little intermission until 4 p. m., as shown in tracings taken at 3:20 and at 4 p. m. (fig. 7). With the more severe spasms, there was an audible inrush of air through the glottis. The animal was restless and apparently attempted to control the spasms by assuming various positions, such as crouching flat to the floor, hopping for a short distance, leaning sharply to the left, or getting up high on its extremities. Moderate uniform pressure over the region of the abdomen checked the spasm for short intervals on repeated occasions, but they recurred immediately as the pressure was withdrawn. At 4 p. m., 2 cc of my anti-encephalitis serum was injected subcutaneously. The needle prick provoked generalized clonic spasms, and

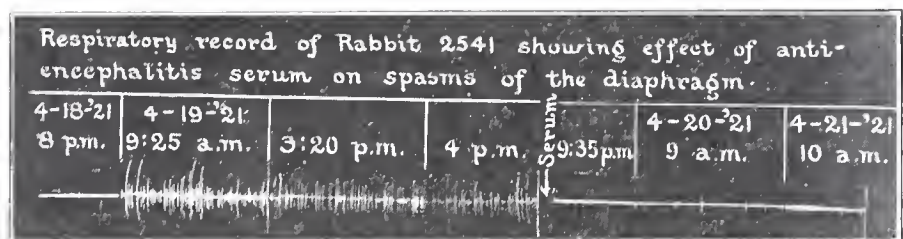


Fig. 7.—Respiratory records of rabbit 2541 injected intracerebrally with the salt solution suspension of pus from the tonsil; effect of the anti-encephalitis serum on the spasms of the diaphragm.

the spasms of the diaphragm recurred almost immediately after the cessation of the general spasm. At 5:20 p. m., the spasms of the diaphragm were less severe and less frequent, and the animal appeared more comfortable. At 6 p. m., the spasms of the diaphragm had largely disappeared. Serum was again injected. Immediately after injecting the serum, generalized clonic spasms again occurred. At 7:30 p. m., the spasms of the diaphragm had nearly ceased and at 9:35 p. m., they had entirely disappeared (fig. 7). At 10:30 p. m. animal seemed better and it was given 60 cc of carrot juice by stomach tube. It ate carrot placed in its mouth. April 20, at 6:45 a. m., the animal appeared well, although it was hyperesthetic and tremulous, and excitation caused twitchings of the muscles of the fore extremities. There were no spasms of the diaphragm. At 9 a. m., an occasional slight spasm of the diaphragm occurred (fig. 7). Carrot juice and corn meal were given by stomach tube, and the serum injection was repeated. While a motion picture was being taken the animal became excited and restless, and developed marked tremor of the entire body. During repeated observations throughout the day, and late into the night, only an occasional slight spasm of the diaphragm was noted, always occurring after prodding or when the animal attempted to walk.

April 21, at 10 a. m., the animal appeared quite well. It lay quietly in the cage, with slight tremor of the head and an occasional slight spasm of the diaphragm. When forced to hop, marked tremor of the extremities became apparent, and it drew its head sharply downward, first to the right and then to the left, as it attempted to bite the fur on the fore extremities. It refused carrot which was held before it; when carrot was placed in its mouth, it chewed, but seemed afraid to swallow. Sixty cc of carrot juice and corn meal were given with a stomach tube, and the serum injection was repeated. The condition of the animal during the day and late into the night remained about the same. Occasional mild spasms of the diaphragm occurred on excitation.

April 22, at 9 a. m., the animal appeared better. It had occasional clonic spasms of the muscles of the neck and fore extremities, and slight spasms of the diaphragm. It still seemed afraid to eat, and mastication of carrot caused clonic spasms of the muscles of the eyes. It frequently drew its head under its chest and between its legs, grinding its teeth violently. Sixty cc of carrot juice and corn meal were again given by stomach tube, and the serum injection was repeated. The passing of the stomach tube provoked clonic spasms of the muscles of the fore extremities and of the neck, and the animal turned its head sharply under its body. At 11 a. m., sharp twitchings and tremor of the fore extremities occurred, and it continued to turn its head sharply downward. The animal became exhausted during the taking of a motion picture. At 3 p. m., it was found dead.

The vessels of the meninges were moderately congested; a portion of the cerebellum on the left side was chocolate colored and edematous. There were no gross lesions in the brain, cord, diaphragm, phrenic nerve, nerve trunks or viscera, and no infiltration, hemorrhage, or edema at the point of injection of the serum.

Cultures from the brain yielded a few colonies of the green-producing streptococcus; those from the blood, kidney, liver, and spleen remained sterile. Sections showed round-cell infiltration of the pia, especially marked surrounding the blood vessels, and adventitial and perivascular infiltration of the vessels in the basal nuclei and in the gray matter of the cervical cord.

A rabbit injected with 0.1 cc of the primary culture from the brain of this rabbit developed tremor and twitching of the muscles of the anterior part of the body, weakness of extremities, rigidity of the muscles of the jaw, inability to swallow, and peculiar motion of the head, slow rotation to the left, synchronous with vertical nystagmus.

Rabbit 2543, weighing 2000 gm., was injected intracerebrally April 16, 1921, with 0.2 cc of a salt solution suspension of the pus from the tonsil diluted 100 times. April 17, at 7:30 a. m., the animal was somewhat excitable and had developed peculiar sudden lateral movements of the head, due to spasms of the muscles of the neck and shoulders. At 2 p. m., it was more restless, had developed tremor of the anterior part of the body, and repeatedly turned its head sharply to the right and under its body. At 6 p. m., it was tremulous and hyperesthetic and had occasional horizontal nystagmus synchronous with lateral movements of the head. Sometimes it turned its head under its body. Sharp clonic spasms of the muscles of the ears and fore extremities had developed and were so severe that the animal at times almost fell over. At 9 p. m., the condition was about the same. There were no spasms of the diaphragm.

April 18, at 7 a. m., there was marked tremor of the head and the fore part of the body. The animal was restless, and ataxic, and had regularly timed

clonic spasms of the diaphragm of irregular severity. Both fore and hind extremities were weak. At 9 a. m., it had rhythmic spasms of the diaphragm, varying in intensity (figs. 8 and 9), and with the more severe spasms, there was an audible inrush of air through the glottis. The effects of ether anesthesia and heroin on the spasms were studied. The animal was anesthetized 3 times during the course of an hour. The first 2 times the tambour recorded its struggles during the stage of excitation; the third time it was anesthetized without a struggle (fig. 8). In each instance the spasms disappeared as anesthesia became complete, but returned promptly when the ether was discontinued. The spasms continued unchanged until 1:30 p. m. (fig. 9), when,

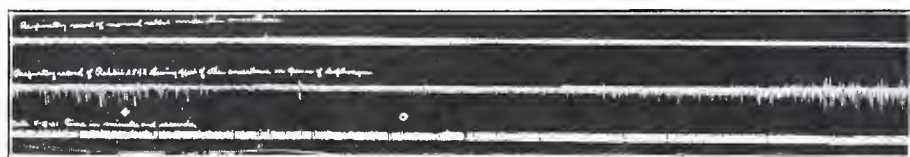


Fig. 8.—Respiratory record of rabbit 2543, showing effect of ether anesthesia on spasms of the diaphragm.

during the course of 30 minutes, 4 intravenous injections of 0.054 mg. heroin hydrochlorid were given. At 2 p. m., the animal drank water; it was less restless and the spasms had ceased (fig. 9). At 3:40 p. m., occasional slight spasms of the diaphragm recurred. At 8:45 p. m., the respirations were extremely rapid, varied greatly in rate and amplitude, and the spasms recurred at intervals (fig. 9).

April 19, at 1 a. m., the respirations were still rapid and irregular. The animal was restless, tossed its head from side to side, and attempted to hop, but was unable to do so on account of weakness. At 4 a. m., it was

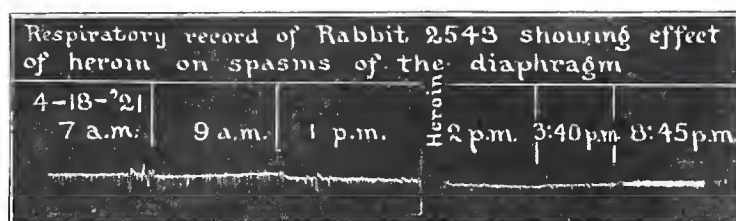


Fig. 9.—Respiratory record of rabbit 2543, showing effect of heroin on spasms of the diaphragm.

still restless and respirations were very irregular, but there were no spasms of the diaphragm. At 7:30 a. m., when the animal was lifted to a table for a kymographic tracing, it had generalized clonic spasms which recurred at 7:40 a. m.; the respirations became shallow, and there were occasional twitchings of the muscles of the fore extremities. At 8 a. m., a violent generalized convulsion occurred. At 8:30 a. m., it lay quietly on its side breathing slowly; it had frequent sharp spasms of the muscles of the fore and hind extremities, and occasional spasms of the abdominal muscles and diaphragm. One cc of normal horse serum, injected intravenously, was without effect on the muscular spasms. At 10:30 a. m., the animal died and was examined at once.

The vessels of the meninges were markedly congested, and the cerebrospinal fluid was slightly turbid. A number of small hemorrhages were found between the cerebellum and medulla. There were no lesions in the phrenic nerve, the spinal nerves, nerve trunks, or diaphragm.

Smears from the surface of the brain showed a moderate number of leukocytes, round cells, and gram-positive diplococci. Cultures of the brain and cerebrospinal fluid yielded countless numbers of the characteristic streptococcus; those from the blood, liver, kidneys, testicle, bile, and lungs remained sterile. A filtrate from the brain emulsion was also sterile, and was without effect when injected intracerebrally into two rabbits.

The culture of the streptococcus isolated from the brain of rabbit 2543 was rapidly transferred in glucose-brain broth. In the ninth subculture the strain was inoculated into devitalized teeth of 1 dog and injected intracerebrally into 2 rabbits. Early the following morning the dog appeared well, but later in the day it seemed afraid to move, held its head in an extended position, and had repeated sharp clonic spasms of the muscles of the neck. The same symptoms were noted during repeated observations the following day, after which the animal recovered. There was no noticeable swelling opposite the roots of the infected teeth, which remained firmly in place.

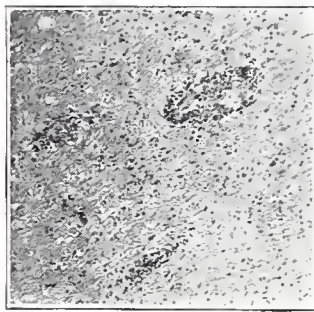


Fig. 10.—Perivascular infiltration in subcortical region of left cerebral hemisphere of a dog 11 days after intracerebral inoculation of strain 4281. Hematoxylin and eosin; $\times 1000$.

One rabbit received 0.1 cc of a 1:100 dilution of the glucose-brain-broth culture, the other 0.1 cc of the undiluted culture. The former was observed from April 2 (the day of injection) to July 12, when it was chloroformed for examination. On the third day after injection it became ataxic and had occasional spasms of the diaphragm. The ataxia became gradually less marked as rotation of the head, increased tonus of muscles, and lethargy developed. At the end of a month it had completely recovered, except for marked rotation of its head, which was held in an almost horizontal plane.

Cultures from the brain, the cord, and the blood were negative, and sections of the cerebrum, pons and medulla showed slight adventitial and perivascular round-cell infiltration in the subcortical region and in the pia, and slight neurophagocytosis in the gray matter of the cord. A few gram-positive diplococci were found adjacent to areas of infiltration.

The rabbit receiving 0.1 cc of the undiluted culture died during the night of cerebral hemorrhage and hemorrhagic edema of the lungs. One and five-tenths cc of the primary culture from the brain, which yielded a pure growth of the streptococcus, was injected intracerebrally into 1 dog. It developed

clonic spasms of the muscles of the neck and ears, twitchings of the muscles of the extremities, increased tonus of muscles, and moderate ataxia, and died 11 days after injection.

The cerebrospinal fluid was slightly turbid, and the pia slightly cloudy. There were no lesions in the viscera. Cultures from the blood and brain yielded a pure growth of the green-producing streptococcus, and sections showed perivascular round-cell infiltration in the basal ganglia, in the sub-cortical area (fig. 10), in the medulla and in the pia.

Three rabbits were inoculated intracerebrally with the brain emulsion of this dog, each receiving 0.1 c.c., one the undiluted culture, another 1:10 dilution, and the third 1:1000 dilution in salt solution. All died of suppurative meningitis, with countless numbers of hemolytic streptococci in the spinal fluid and blood. One and five-tenths c.c. of the filtrate of the brain emulsion was injected intracerebrally into each of 2 rabbits. Both remained well.

CASE 4.—Mr. F. U., aged 35 years, entered the Clinic Feb. 2, 1922, complaining of persistent hiccup. He had had a "slight cold" late in November, about the time the attack of hiccup began suddenly and while caring for his mother, during an attack of encephalitis. During this attack she had had recurring spasmodic, jerky attacks, involving the legs, arms, face and nearly the whole of the body, which lasted for 8 or 9 weeks, following an attack of fever. She had had no previous illness. The patient's attack consisted of a short series of rapidly repeated, audible, jerky, spasmodic inspirations, occurring about every 15 minutes, followed by intervals of apparently normal respirations. At first the attacks recurred many times during the day and often kept the patient awake at night, but in the last few weeks they had become fewer and less severe. At onset, fever and sore throat were not present. The appetite had been good, and the bowels regular. There was complete absence of vomiting, soreness, and pain in the abdominal muscles, difficulty in swallowing, food distress, cough, and difficulty in breathing. The blood pressure was normal. Examination of the blood showed 8,800 leukocytes and hemoglobin 75%; the Wassermann test was negative. A sample of urine showed a trace of albumin, but otherwise was negative. Physical and roentgen-ray examinations of the chest were negative. General and neurologic examinations showed no abnormalities except hiccup. The patient was given an emulsion of benzyl benzoate. The nasopharynx was hyperemic and moist; the tonsils were small and no pus was expressed. A fluoroscopic examination by Dr. Carman proved that the hiccup occurred during sudden contractions of the diaphragm, and since the patient was able voluntarily to bring on these attacks during this examination, a diagnosis of hysterical hiccup was made.

Of the 17 animals injected intracerebrally in this case with the streptococcus from the nasopharynx, irrespective of the number of subcultures and animal passages, 12 developed twitchings or clonic spasms of the muscles as the striking symptom, and 7 had marked spasms of the diaphragm. Ten developed tremor, 4 nystagmus, 1 lethargy, and 9 paralysis of varying degrees (table 1). As this streptococcus was passed through animals its localization changed. As spasms of the diaphragm became less common the incidence of paralysis increased. The spasms of the diaphragm occurred in 5 and paralysis developed in 2 of 6 rabbits injected with the strain in the first and second animal passages, while spasms of the diaphragm developed in only 2 and paralysis in 7 of 11 rabbits injected in the third and fourth passages.

CASE 5.—Mr. S. J. H., aged 35 years, developed persistent hiccup Feb. 19, 1922, several days after having had a mild sore throat. The spasms of the

diaphragm were relatively mild but were often accompanied by audible hiccup, and generally continued for 48 hours. The usual anodyne treatment failed to control the spasm. On the second day, during preparations for a respiratory tracing, the spasm suddenly ceased, but recurred several times during the day and then entirely disappeared. Aside from slight burning in the throat, the patient felt well. The leukocyte count was 10,800, and a blood culture proved negative. The temperature was 99, and the pulse regular and normal in volume.

Physical examination revealed a robust, well nourished, young man with teeth and gums in perfect condition. The nasopharynx was diffusely red and moist. A small amount of pus was expressed from the tonsils, which were small and submerged. In washing out the nasopharynx, a large amount of turbid mucus was obtained.

Of the 16 rabbits injected intracerebrally with suspensions in salt solution of the pus from the tonsils, and nasopharyngeal washings, with the primary cultures, and with brain emulsions after from 1 to 4 animal passages, 8 developed clonic spasms or twitchings of muscles, and 1, spasms of the diaphragm also. Six developed marked respiratory symptoms, 4 marked ataxia,

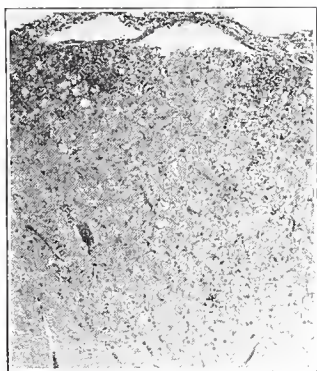


Fig. 11.—Marked infiltration of the pia and adjacent anterior portion of the medulla in a rabbit 4 days after intracerebral inoculation of strain 4589. Hematoxylin and eosin; $\times 50$.

and 5 nystagmus (table 1). The peculiar movements of the head were unusually marked in the animals injected with this strain. The relative infrequency of spasms of the diaphragm in this series of animals and the comparatively mild muscular spasms were in keeping with the mildness and short duration of the attack in the patient. The animal that developed spasms of the diaphragm died on the fourth day. It showed marked leukocytic infiltration in the anterior aspect of the medulla and overlying pia (fig. 11).

One month after these experiments were performed and the patient had recovered, two rabbits were injected with the suspensions of swabs from the tonsils; both remained well.

CASE 6.—Mr. W. J. H., aged 57 years, came to the Clinic March 4, 1922, seeking relief from persistent hiccup. The attacks had begun with severe hiccup when he had wakened one night 6 days before and had continued almost constantly during the day. At first he had been able to sleep at night. Later the attacks, 10 to 20 each minute, had not only continued practically all day, but had kept him awake at night, except for occasional intervals of from $\frac{1}{2}$ to 1 hour. The night previous to examination he had slept for

7 hours without waking, and had been free from hiccup for 4 hours during the day. He had felt weak and exhausted from persistent spasms of the diaphragm. Since the beginning of the attacks he had taken light food, but his appetite had been good. Thirty-two years previously he had had a similar attack lasting 1 week, and 7 years previously he had had a milder attack lasting 2 or 3 days. Since that time at infrequent intervals he had had hiccup lasting about half an hour. Three years previously he had been troubled with cructations of gas and vomiting after meals; this had disappeared after attention to infected teeth.

General examination revealed a few fine, moist râles in the left lower base, normal blood pressure, pulse rate and temperature. Total urea and urea nitrogen of the blood on March 6 were normal, and a culture of the blood remained sterile. Spasms of the diaphragm disappeared during the night before my examination and remained absent. The breath was foul, due to a marked infection of the gums and to caries. The tonsils and nasopharynx were hyperemic and moist and were covered with a moderate amount of mucus.

One rabbit injected with the suspension in salt solution of the swab from the tonsil and nasopharynx died in 24 hours from a hemorrhage in the right lateral ventricle at the point of injection. Three rabbits were injected intracerebrally with the primary culture in glucose-brain broth of a mixture of the suspensions from the throat and tonsil swabs. One received 0.2 cc of the undiluted culture, the second 0.2 cc of a 1:10, and the third 0.1 cc of a 1:100 dilution in salt solution. Platings of the culture injected yielded a large number of indifferent and green streptococci and a few staphylococci. All 3 developed spasms of the diaphragm (table 1).

CASE 7.—Mr. H. S., aged 22 years, was sent to me March 12, 1922, by Dr. Kilbourne, on account of a persistent hiccup which had begun 24 hours previously. The spasms of the diaphragm had continued all day, March 11; they had disappeared between 8 and 9 p. m., recurred between 9 and 10 p. m., but had disappeared on his retiring. He had slept until 4 a. m., when he was awakened by the recurrence of the hiccup. Hoffman's anodyne was given in an attempt to control the spasms. The hiccup had continued until 9 a. m., but ceased as the patient entered the laboratory at 10:30 a. m.

The tonsils were large and red, and contained deep crypts which were not plugged with cheesy material; only a small amount of pus could be expressed. The nasopharynx was hyperemic and moist. The left upper second bicuspid had been crowned two years before, and was somewhat tender on tapping. The spasms of the diaphragm remained absent until supper time, when they recurred for several hours, disappearing on retiring so that the patient slept soundly all night. There were several light recurrences the following morning and during the latter part of the afternoon the attacks became so severe he could scarcely speak and he became very much exhausted. When he came to the laboratory for a tracing, the hiccup was marked, and the skin was pale and moist. The spasms continued, but, as in case 5, they disappeared as the adjustments were made to obtain a tracing. Since some rabbits experimented on developed marked disturbance of respiratory rhythm in addition to spasms of the diaphragm, the tracings in fig. 12 were taken. The arrhythmia noted in the tracings in this case, as in case 3, and the character of the spasms in the latter are identical with those noted in the tracings of Roger and Schulman,³ in their case of epidemic hiccup. Spasms of the diaphragm, therefore, may be only one of several effects on the respiratory mechanism in

³ Presse Med., 1921, 29, p. 161.

epidemic hiccup. The spasms did not recur until the following afternoon, lasting four hours, then entirely disappearing. The patient complained of soreness and pain in the upper abdomen due to the violent spasms. He attributed his attack to blowing a whistle in refereeing a basket ball game and to eating bananas the night before the hiccup began. He could taste bananas during the spasms for 24 hours after eating them.

Suspensions of the tonsil pus and nasopharyngeal swab were each injected intracerebrally into one rabbit. The animals developed almost identical symptoms, of which spasms of the diaphragm were predominant.

A highly diluted mixture of the glucose-brain-broth culture from the nasopharynx and tonsil in the eighth and ninth culture generations, respectively, was injected intracerebrally into 2 rabbits. The one receiving 0.1 cc of a 1:10,000 dilution remained well; the other, injected with 0.1 cc of a 1:1000 dilution developed marked rhythmic spasms of the diaphragm.

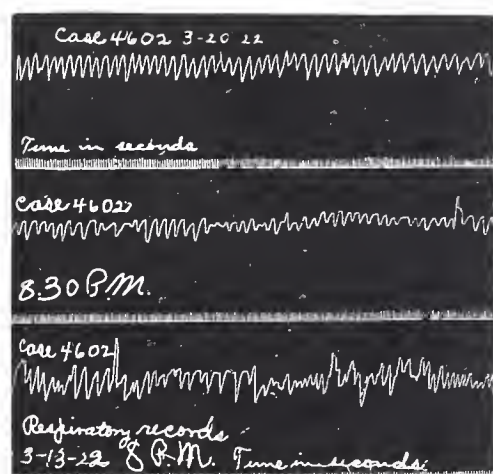


Fig. 12.—Respiratory records of case 7, showing marked arrhythmia immediately following cessation of hiccup attack, improvement one-half hour later, and normal respiration one week later.

Spasms of the diaphragm were observed in 5, and spasms of the diaphragm or other muscles in 8 of the 9 rabbits injected with the freshly isolated streptococcus in this case (table 1).

One week after the attack had ceased the infecting power of the bacteria in the throat of the patient was again studied. Spasms of the diaphragm were not observed in any of 4 rabbits injected.

CASE 8.—Mr. W. J. S., aged 20 years, was referred to me April 8, 1922, on account of an attack of hiccup. This began without apparent cause during the afternoon, 2 days previously. The patient had a "cold" in the head for 2 days prior to the development of hiccup. July, 1921, he had had a similar attack lasting for 2 days. The patient's father at that time also had had hiccup which lasted for 5 days. The spasms varied greatly in time and intensity; they continued with short interruptions during the day, but the intervals between attacks were longer at night. During the 3 attacks of the night previous to my examination, the spasms continued until the patient vomited.

The mucous membrane of the nose and pharynx was hyperemic and covered with a moderate amount of tenacious mucus. The tonsils had been cleanly removed. The leukocyte count was 9,500, the temperature normal. Spasms of the diaphragm were absent at the time of examination but occurred that afternoon and the next day for a short time, and then ceased. The blood-agar plates of the throat swab showed a large number of colonies of indifferent and green-producing streptococci, a few staphylococci and *Mitococcus catarrhalis*, and no hemolytic streptococci.

The salt solution suspension of the nasopharyngeal swab was injected intracerebrally into 4 rabbits, 1 with 0.1 cc, and 3 with 0.2 cc of a 1:1000 dilution. Three developed tonic spasms of various muscles, and tremor and twitching of the masseters and other muscles; two also had spasms of the diaphragm. The fourth, which received 0.1 cc of the 1:1000 dilution, remained well.

A mixture of the primary 14-hour culture of the brain of the 2 rabbits that developed spasms of the diaphragm was injected intracerebrally into 2 rabbits. One was given 0.1 cc of the undiluted mixture and died over night from meningitis. The other was injected with 0.1 cc of a 1:100 dilution and

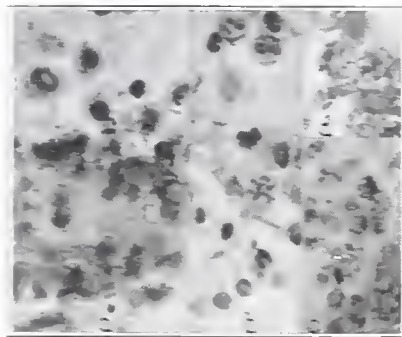


Fig. 11.—Various sized diplococci and coccic forms in lesions of the central nervous system illustrating that developed spasms of the diaphragm: a blood vessel showing perivascular infiltration containing large and medium sized diplococci above, and the large coccic form in germinal zone below; b, medium sized diplococci and short chain in leukocytes in area of inflammation shown in fig. 10; c, diplococci within and outside of leukocytes showing various stages of degeneration in area of necrosis; d, small, medium sized and very large diplococci in a small space from the middle of the posterior horn of the ventricle in a rabbit that developed spasms of the diaphragm following intracerebral injection of the bacillus streptococci from case 1. (Gram-Walsby, ×1000.)

developed fine and coarse tremors and short spasms of the muscles of the fore part of the body, occasional spasm of the diaphragm, tonic contraction of the masseters, and an ascending paralysis. Both yielded pure cultures of the characteristic streptococcus in countless numbers from brain and spinal fluid.

SUMMARY OF RESULTS

Soon after these experiments were begun it became apparent that the animals developing marked spasms of the diaphragm following intracerebral inoculation often died, and that symptoms of encephalitis developed before death. Table 1 gives a summary of the incidence of the more important symptoms. The incidence of spasms of the dia-

phragm and total incidence of muscular spasms varied greatly; in general, they were highest in the patients having the longest attacks or the most severe spasms. Thus, spasms of the diaphragm occurred in 5, and muscular spasms in 8, of the 9 animals injected with the strain from case 2 in which the spasms were severe and persisted at intervals for six weeks. Spasms of the diaphragm occurred in only one and muscular spasms in 8 of 16 animals injected with the strain from case 5 in which the spasms were mild and lasted only 2 days. In summarizing the results in the 8 cases studied, we find that about 40% developed spasms of the diaphragm, and about 74% of the 96 animals injected developed muscular spasms, including spasms of the diaphragm. This included animals injected intracerebrally with suspensions of material obtained directly from the throat, or tonsils of the patient, with cultures from these, with emulsions and fresh cultures of the brain of animals, with positive cultures of the streptococcus from filtrates, and with cultures after many rapidly made subcultures.

During the experiments, these strains tended to produce lethargic symptoms, especially after a number of animal passages and after many rapidly made subcultures, provided the dose was properly gauged. It was thought worth while, therefore, to determine the incidence of this and other symptoms in the animals injected with freshly isolated strains in the first and second animal passages, and in those injected after 2 or more animal passages and after many rapidly made subcultures. There were 45 animals in the first group and 51 in the second (table 1). Of the 45, 26 (58%) developed spasms of the diaphragm, and 39 (86%) developed muscular spasms, while only 4 (9%) developed lethargy. Of the 51, only 14 (27%) developed spasms of the diaphragm; and 32 (62%) developed muscular spasms, while 17 (33%) developed lethargy. The tendency of the streptococcus to produce lethargy was more marked in the 3 cases studied in 1921 than in the 5 studied in 1922. In the former group this symptom developed in 16 of 44 animals injected; in the latter, in only 5 of 52 animals. The increased incidence of rhythmic movements, usually synchronous with respirations, in the second group over that in the first is noteworthy.

The figures in the table do not adequately express the changes observed in the localizing power of the streptococci on intracerebral inoculation. If the dose was properly gauged, spasms of the diaphragm and other muscles were often produced in duplicate for several successive animal passages even after many rapidly made subcultures. In some instances, for indefinite reasons, this was not possible. If the

dose on successive animal passage was made relatively large, it lost the power of producing spasms of the diaphragm and caused rapid death from diffuse meningitis, especially if the brain emulsion was inoculated directly and if death occurred early. This was also true after prolonged cultivation in glucose-brain broth. Thus, one strain under constant dosage produced characteristic symptoms in the primary culture in the 7th, 33d, 37th and 50th subcultures, while in the 73d subculture spasms of the diaphragm were not produced, but suppurative meningitis developed. If the dose was small or if the strain was from animals that were recovering, lethargic symptoms often developed, or the strain lost the power of producing symptoms of any kind. The tendency to acquire certain new localizing powers was often apparent in each of a series of animals, although exceptions, due to differences in resistance in the animal or some other factor, were not uncommon.

The inherent localizing power of the streptococcus, however, was clearly the most important factor in determining the result even on intracerebral inoculation. Gross lesions at the point of injection in the right frontal lobe were usually absent. Identical symptoms often developed when the place of injection was altered, such as deep injections in the direction of the right lateral ventricle, superficial injections in the right frontal lobe and cistern injections.

Altogether 28 animals were injected intracerebrally with filtrates, 3 of which were prepared from the nasopharyngeal washings, 14 from the brain emulsion of positive animals and 3 from cultures of the streptococcus from positive animals. Five of the filtrates yielded the streptococcus in pure culture, the rest were sterile. Four produced positive symptoms in one animal each. One of the animals died in 5 days and a pure culture was isolated from the brain. In another animal which became lethargic the filtrate was prepared from the brain emulsion of a positive animal following injection of the streptococcus after 1 animal passage and 37 subcultures. It showed characteristic lesions, and streptococci were recovered from the brain. The remaining 2 were chloroformed in 12 and 21 days, respectively, cultures from the brain remaining sterile. Sections revealed slight perivascular and localized infiltration in the pons. Cultures and sections were negative in 10 of the animals without symptoms which were chloroformed in from 7 to 21 days after inoculation.

Similar, although less striking, results were obtained by other methods of inoculation of these strains. Characteristic symptoms or lesions were obtained in 3 of 8 rabbits injected intravenously; in 1

of 4 following the packing of the nose with gauze soaked in cultures, and in three animals, 2 dogs and 1 rabbit, in which teeth were devitalized and infected with cultures of the streptococcus from positive animal. Negative results were obtained in a study of the infecting power of bacteria from the teeth and throat in a patient who developed persistent hiccup and vomiting following an exploration for cerebellar tumor. In 3 of the patients tests were made of the infecting power of the bacteria obtained in the same way 7, 10 and 30 days, respectively, after recovery. Only 1 of 9 rabbits injected developed spasms of the diaphragm. In the others either the strain had disappeared or had lost this peculiar power, because these strains rapidly lose the power of producing spasms of the diaphragm unless subcultures are made from 4 to 6 times a day, under reduced oxygen tension. One aerobic plating may be sufficient to destroy this property. I have attempted unsuccessfully to preserve this property indefinitely in various ways. Viability was preserved for a long time in the dried brains of positive animals, in deep tubes of meat infusion and glucose-brain broth, on blood-agar slants and in ascites-tissue fluid, but the ability to produce spasms of the diaphragm had disappeared in all within 2 months after isolation and in most instances in much less time. Spasms of the diaphragm did not occur in a single instance following inoculation either of fresh transplants or of the old cultures into 47 rabbits.

Diaphragmatic spasms were not observed in 61 rabbits injected intracerebrally with material from the throats of 63 normal persons, most of whom had been in contact with patients with poliomyelitis during the epidemic of poliomyelitis in and about Rochester in the summer of 1921. Spasms of the diaphragm developed in only 2 of 92 rabbits injected intracerebrally with material from the throats of 49 patients with poliomyelitis during the same epidemic. The 2 positive instances occurred in the first and second animal passages following injection of streptococci from the throat of one of the patients 2 weeks after onset of the attack. Moreover, the incidence of spasms of the diaphragm in a large series of rabbits with injections from the throat and tonsils in cases of lethargic and other forms of encephalitis is much smaller than that obtained with the hiccup strains.

Cultures after death were made in 81 animals which showed symptoms when chloroformed or which died as the result of the injection. The characteristic streptococcus was isolated from the brain in 76 and from the blood in 31. Other bacteria, chiefly *B. coli*, were isolated in 8. This is in sharp contrast to the results of the cultures made in 28

animals that remained free from symptoms or had recovered and were chloroformed for examination. Streptococci were not isolated in any instance from brain or blood, and the colon bacillus was found in the cultures from the blood in only 1.

The nasopharynx of all the patients was abnormally moist and hyperemic. The tonsils were of normal size in 4, enlarged, and badly infected in 3, and absent in 1. A variable amount of pus was expressed from the tonsils in 7. Positive results were obtained in animals inoculated with strains from the tonsils of all but 1 of 7 patients and in all of 7 animals in which injections were made with material from the nasopharynx. There was evidence of marked dental infection in 4. The cultures from the pyorrheal pockets in the one yielded negative results.

The epidemic character of the attacks in the patients studied was quite apparent; no evident cause, such as indiscretion in diet, was present. In 3 of the patients the attacks occurred between February and April of 1921, and in 5 during this period in 1922. Three of the patients were from the Rochester State Hospital, 1 an epileptic, 1 a male nurse, and 1 a baker. During the first period, I learned of 14 additional cases of persistent hiccup, and during the second period of 9 others; while between April, 1921, and February, 1922, and since April, 1922, only 2 cases of the same type came to my attention. Two of the patients had had previous attacks. The father of one of the patients had an attack at the same time. In one, hiccup developed while the patient was caring for his mother during an attack of encephalitis in which muscular spasms were a prominent symptom. Subjective symptoms of infection of the upper respiratory tract were noted in 4 a short time before the onset of the attack. The temperature of all was normal.

DESCRIPTION OF THE STREPTOCOCCUS

Blood-agar-plate cultures from the suspensions from the nasopharynx and tonsils in 7 of the patients showed a great preponderance of colonies of green-producing streptococci, and a moderate number of staphylococci and *Micrococcus catarrhalis*. In one the culture from the tonsil yielded countless numbers of colonies of hemolytic streptococci, while those from the nasopharynx yielded chiefly green-producing streptococci.

Direct platings of the material obtained from the infection atriæ were not characteristic, aside from the fact that the green-producing

streptococci were numerous, and injections of animals with cultures from single colonies did not produce spasms of the diaphragm. Direct inoculation of the suspensions of the material in sodium chlorid solution and of mass cultures of these in tall columns of glucose-brain broth was necessary to reproduce this striking symptom.

The animal was made to serve as a plate. Saprophytes disappeared promptly, and the characteristic streptococcus was isolated in pure or nearly pure cultures from the brain of the animals showing spasms of the diaphragm. The colonies in all of the strains were similar; they were dry, nonadherent, slightly elevated, sometimes umbilicated with smooth margins and surrounded by a narrow, greenish zone. They resembled group 4 pneumococci and so-called *Streptococcus viridans*. The colonies of some of the strains became larger and more moist after repeated animal passage. In glucose-brain broth most of the strains produced diffuse turbidity suitable for agglutination. This was not true in most of the strains after prolonged cultivation and in those from the throats after recovery.

Smears from young cultures in glucose-brain broth showed gram-positive, nonencapsulated, usually elongated diplococci of quite uniform size and shape arranged singly and in short chains. The virulence, as measured by the usual methods of inoculation, was low. Intraperitoneal injection in mice and intratracheal injection in guinea-pigs of large doses (0.5 to 1.5 cc) of the culture were usually ineffective.

The fermentative power over the usual carbohydrates, including inulin, was determined in 24 subcultures of 7 of these strains. One or more subcultures soon after isolation of 4 of the strains fermented inulin, while 3 did not. The amount of acid produced in inulin, determined by the depth of the red color of the fuchsin used as indicator, varied greatly in the different strains. In 2 strains, it was either absent or slight in each of the subcultures soon after isolation. In one of these, acid production became marked after the strain had passed through 63 subcultures in glucose-brain broth. Two of the strains which failed to ferment inulin soon after isolation acquired this power after cultivation on artificial mediums for some months. In 2 strains acid production in inulin was marked in all of the tests both early and late. Dextrose, lactose, maltose, and saccharose were fermented by all of the 24 cultures studied, raffinose by 20, and salicin by 18. In mannite and the controls, acid production was absent in all. The blood-agar plates made at the same time showed a pure culture of typical green-producing colonies in each instance. Most of the cultures tested were

insoluble in bile. Agglutination experiments with my antipoliomyelitis and anti-encephalitis serum, with a serum prepared with one strain of green-producing streptococcus from epidemic influenza, with anti-pneumococcus serums, types 1, 2, and 3, and with normal horse serum, were made with all these strains when isolated after one or more animal passages. Specific agglutination of the primary culture of the material obtained from the infection atrium occurred in the polioencephalitis serums in 2 cases; while in the strain from positive animals this occurred in 5 cases; none of the strains were agglutinated specifically by any of the other serums.

SYMPTOMS IN EXPERIMENTAL AND SPONTANEOUS HICCUP

The symptoms in the animals that recovered often consisted only of spasms of the diaphragm, whereas in those that succumbed to intracerebral inoculation, generalized myoclonic movements, marked disturbance of the respiratory rhythm, and other symptoms of a more widespread encephalitis often supervened. In the former, the microscopic lesions were relatively slight and usually limited to the pons, medulla, and upper cervical cord, while in the latter, they were often marked and widespread. The disease produced experimentally thus resembled the findings in human beings, for in the fatal cases of the spontaneous disease spasms of muscles and grave respiratory disturbances develop in addition to spasms of the diaphragm; similar widespread lesions have been found in the brain, medulla, and cervical cord.⁴ The disease produced experimentally resembled that noted clinically in still other respects. The time of occurrence of the spasm in relation to the respiratory cycle, the audible inrush of air through the glottis in the more severe spasms, the irregularity in rate and severity of the spasms, and the changes in respiratory rhythm were similar in the experimental and spontaneous disease. Procedures, such as firm pressure over the abdomen, on the neck, and over the course of the phrenic nerve, and respiratory depressants, such as heroin, tended to control the spasms temporarily. The anti-encephalitis serum controlled them permanently in an animal inoculated with one strain which was agglutinated specifically by the serum. The character of the symptoms and lesions produced lends support to the popular view that epidemic hiccup is really a form of the myoclonic type of encephalitis. The clinical and epidemiologic

⁴ DuCamp, Carrieu, Bloquier de Claret and Tzelepoglow: *Bull. d. l'Acad. de méd.*, 1921, 86, p. 249. Furno, A.: *Rif. med.*, 1921, 37, p. 77. Pierre-Kahn, Barber and Bertrand: *Bull. et mém. Soc. med. d. hóp. de Paris*, 1921, 37, p. 787.

findings indicating the similarity in the etiology of epidemic hiccup and epidemic encephalitis⁵ are supported by the occurrence of lethargic symptoms following inoculation of some of these strains and the character of the microscopic changes in these animals, the protective power of the serum prepared in the horse with a single strain of a streptococcus from a case of lethargic encephalitis against infection by the hiccup streptococcus, and the fact that the streptococcus in 5 of the 8 cases was agglutinated specifically by this serum.

GROSS AND MICROSCOPIC LESIONS IN ANIMALS

The phrenic nerve was free from lesions in all cases, as were the vagus and sympathetic nerves and ganglions. The diaphragm in a few animals, always in those having violent spasms, showed localized hemorrhages. These were irregularly distributed and were not grouped about branches of the phrenic nerve. In some of the experiments in which rabbits died soon after intracerebral inoculation, there was marked digestion of the stomach with perforation of the fundus and the diaphragm. This was certainly an antemortem reaction in some of the animals because it was present when necropsy was performed immediately after death, and although the viscera presented cloudy swelling and other signs of overwhelming infection, the blood in all was either sterile or contained relatively few organisms. It appeared to be a reaction central in origin. Lesions of other viscera were rarely observed.

The lesions found after death, studied in relation to the symptoms observed during life, showed that spasms of the diaphragm may result from lesions in or surrounding the anterior roots of the upper cervical nerves corresponding to the origin of the phrenic nerve, in the anterior part of the upper cervical cord or medulla, in the pons, or in the cerebral cortex. That the impulse provoking the spasms is of central origin is also indicated by the fact that heroin, a depressant of the respiratory center, was found to control the spasms. The character of the microscopic changes varied greatly, depending on the duration of the experiment, size of dose, virulency of the streptococcus and the species of animal injected. In the animals that died early, leukocytic infiltration was predominant, while in those that died late, mononuclear cells, some with eccentrically placed nuclei, or plasma cells predominated. The reaction incited was chiefly perivascular in location. Edema and cellular

⁵ Lhermitte, J.: *Presse méd.*, 1920, 28, p. 916. Netter, A.: *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1921, 37, p. 46. Rivet, L., et Lipschitz, M.: *Ibid.*, p. 8.

infiltration were often limited to and always more marked around blood vessels than elsewhere. This was true in the pia as well as in the brain and cord substance. Neurophagocytosis and degeneration of ganglion cells were slight or absent. The walls of the blood vessels were usually free from lesions. The endothelial cells of the intima were occasionally swollen. Accumulation of leukocytes within the vessels, and capillary thrombosis were rare. Localized hemorrhages and infiltrations and, more rarely, areas of focal necrosis were relatively slight and were often adjacent to blood vessels showing perivascular infiltration. The demonstration of the streptococcus in the lesions in animals that succumbed early was relatively easy, while in those that had recovered and were chloroformed it was difficult or impossible. The organisms varied greatly in size and were often found within cells (fig. 13 a, b, c and d). The possibility that perivascular infiltration in the brain of rabbits occurs spontaneously has been effectively ruled out.⁶ The character of lesions observed in the rabbits paralleled the duration of the experiment. The location and extent of the lesions were often what would be expected, judging by the symptoms in the animals. Sections were made of the brains of a large number of control rabbits, many of the same group as those in which characteristic lesions were found. The brains of 14 rabbits that remained free from symptoms following control inoculation with sterile brain emulsions and filtrates, and sterile broth and salt solution were found free from lesions. In the examination of the brains of 64 rabbits which remained without symptoms following inoculation of spinal fluid, filtrates, and other material from cases of encephalitis, lesions were not found in a single instance resembling those of the positive experiments. Only 2 of 53 rabbits whose brains were examined after intravenous inoculation with streptococci from other sources, showed perivascular infiltration in the brain. In these no bacteria were demonstrable. The infiltration was more dense and more sharply circumscribed, and the cells were all mononuclear. Moreover, the spontaneous lesions, as reported in rabbits, were free from demonstrable microorganisms. In the positive experimental animals gram-staining diplococci were demonstrable in the lesions. Finally, all doubt of the ability of these strains to incite these lesions is removed by the fact that they were produced and the organisms demonstrated in the lesions in the monkey and the dog, animals in which perivascular infiltration does not occur normally.

⁶ Oliver, J.: *Jour. Infect. Dis.*, 1922, 30, p. 91.

CONCLUSIONS

The cases studied were undoubtedly examples of epidemic hiccup. From the infection atrium of each a streptococcus alike in morphology and cultural character was isolated, and with each strain spasms of the diaphragm, and other muscles were reproduced in animals. The organism was isolated from these animals and characteristic symptoms again induced on reinoculation. The streptococcus was demonstrated in the lesions and proved absent elsewhere on microscopic examination of sections. These results were not obtainable with streptococci from similar sources in other diseases. The possibility of an accompanying filtrable virus was excluded by the filtration experiments and by the successful reproduction of the disease after many rapidly made subcultures of the different strains. The methods with which positive results were obtained included procedures in which the conditions in the patient were closely simulated. The type of the disease and lesions induced were similar to those noted in the spontaneous disease in man. The conclusion that epidemic hiccup is due to a streptococcus having peculiar neurotropic properties seems warranted.

The question of the origin and absolute specificity of this streptococcus remains to be determined. The facts that it belongs to the pneumococcus-streptococcus group of organisms normally present in the upper respiratory tract of man, that contagion is usually not demonstrable in this disease, and that the power to produce this condition disappears as new and distinctive invasive powers become manifest from successive animal passage and artificial cultivation, suggest that the peculiar localizing power of the hiccup strain is an acquired property, a phase perhaps in the life cycle of the pneumococcus-streptococcus group of organisms.

PRODUCTION OF SPASMS OF THE DIAPHRAGM IN ANIMALS BY LIVING CULTURES, FILTRATES, AND THE DEAD STREPTOCOCCUS FROM EPIDEMIC HICCUP

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During the course of certain experiments on immunization with the streptococcus from epidemic hiccup, it was found that rabbits which were injected intracerebrally with dead bacteria and filtrates of cultures developed spasms of the diaphragm. The significance of this observation, particularly with regard to the mechanism of localization of bacteria, was at once apparent, and the series of experiments reported herewith was undertaken.

In previous studies,¹ the etiologic relationship of a streptococcus to epidemic hiccup has been demonstrated. The condition was reproduced in animals by intravenous and intracerebral inoculations of live cultures, by packing the nose with gauze soaked in the culture, and by the devitalization and infection of teeth. I shall report here the results obtained in a study of the localizing and symptom-producing power of live cultures and the corresponding filtrates and dead bacteria.

TECHNIC

The technic for the early experiments consisted of growing the streptococcus directly from the brain of the animals that had spasms of the diaphragm after inoculation with live cultures in tall columns of glucose-brain broth, glucose broth, and meat infusion, with and without the addition of ascites fluid (in proportions of about 1:10). Some tubes were layered with paraffin oil. Positive results have been obtained with all of these mediums, but glucose-brain broth and meat infusion gave the best results; hence, most of the experiments were made with these culture mediums. The glucose-brain broth was prepared from dehydrated broth (Digestive Ferments Company) by adding 8 gm. of salt solution and 2 gm. of glucose for each liter. This was placed in tall test tubes (20 by 1.2 cm.); pieces of fresh calf brain, totaling about 2 gm., and several pieces of marble were added to the bottom of each tube, bringing the column of medium to 12 cm. The titration after autoclaving approximated $+1.0$, the hydrogen-ion concentration 6.5.

The meat infusion medium was prepared in the usual manner, brought to a hydrogen-ion concentration of 7.8 before autoclaving, and was placed, unfiltered, in tall tubes containing the ground meat and without peptone or sodium chlorid. This medium was used in the hope that the hiccup strains might

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¹ Rosenow, E. C.: Jour. Am. Med. Assn., 1921, 76, p. 1745.

retain specific infecting power, because I had found that pneumococci and streptococci from other sources retained viability and original cultural properties for a long time. The cultures were incubated at 33 to 35 C. The effects of the live culture of the clear centrifugalized culture and filtrate and of the dead bacteria were studied on intracerebral inoculation in rabbits weighing from 1,200 to 2,000 gm., in guinea-pigs from 350 to 500 gm., and in monkeys weighing about 2 kg. The dose ranged from 0.1 cc of the live culture to 0.1 cc of 1:1000 dilutions of the live culture; from 0.5 to 1.5 cc of the centrifugalized culture and filtrates, and the dose of the washed dead bacteria consisted of the growth of from 2 to 5 cc of the broth culture suspended in one-tenth that amount of salt solution. Tests were usually made when the cultures were from 12 to 72 hours old, although in some instances not until after 3½ months. Control injections were made of the uninoculated broth of the same batch, filtered and unfiltered, and of the salt solution used to dilute the filtrate and to suspend the bacteria for injection. Bacterial filters of the Mandler type were used in the preparation of the filtrates; suction from a water pump was sufficient to produce slight bubbling.

The animals were examined frequently and graphic respiratory and other records were made in many. Animals were chloroformed at various intervals following inoculation for examination for gross lesions, for microscopic study of stained smears of spinal fluid and sections of brain and cord, and for control cultures.

EXPERIMENTS

Various cultures of 3 strains of streptococci from epidemic hiccup (cases 4, 7 and 8) have been studied in this manner, and as controls one strain from a case of respiratory arrhythmia with alternating spells of hyperpnea and apnea (strain 4564), and one from a case of acute encephalitis associated with convulsions (strain 4605). The production of spasms of the diaphragm and other muscles with recently isolated live cultures in the 3 hiccup strains is described elsewhere.²

The effect on intracerebral injection of filtrates, dead bacteria, and the corresponding live cultures of the strain from the patient in case 8 were studied in 4 series of experiments. The first series of animals was injected with material from a mixture of equal parts of 24-hour glucose-brain-broth and ascites-glucose-broth cultures. These mediums were inoculated directly with the brain emulsion of 2 rabbits that developed spasms of the diaphragm following injection of the salt solution suspension of the swab from the nasopharynx and primary culture in glucose-brain broth, respectively (table I).

It will be noted that the one rabbit (rabbit 3622) injected with the live culture developed spasms of the diaphragm, tremor, and spasms of the masseters and other muscles. The freshly prepared, unheated filtrate produced spasms of the diaphragm, tremor, and twitchings of the masseters in 3 of 4 animals injected, in both rabbits (rabbits 3623 and 3533), and in one of 2 guinea-pigs (guinea-pigs 2227 and 2228). One guinea-pig developed only twitchings of the masseters and other muscles. The fresh filtrate neutralized produced marked spasms of the diaphragm and other muscles in the one rabbit injected (fig. 1 and protocol of rabbit 3507) and tremor, twitchings of the masseters and muscles of fore extremities and nystagmus in the monkey (monkey 311).

Charcoal removed completely the power of the filtrate to produce spasms of muscles. Two rabbits injected remained free from symptoms (rabbits

² Rosenow, E. C.: Jour. Infect. Dis., 1923, 32, p. 41.

TABLE 1

RESULTS ON INTRACEREBRAL INOCULATION OF THE LIVE CULTURE, THE BROTH CULTURE FILTRATE, AND DEAD BACTERIA OF STRAIN 4621 (CASE 8)²

Material Injected	Dose, C c	Animal*	Result
Living streptococcus.....	0.1 1:100	R3622	Spasms of the diaphragm, tremor and spasms of the masseters, of muscles of neck, ataxia, paralysis of liver and extremities; death
Fresh, unheated filtrate..	1.0	R3623	Spasms of the diaphragm, tremor and twitchings of the masseters and other muscles; lethargy following second injection; recovery
Fresh, unheated filtrate..	1.0	R3533	Moderate spasms of the diaphragm; tremor and twitchings; recovery
Fresh, unheated filtrate..	0.4	P2227	Tremor and twitchings of the masseters and other muscles; death in 24 hours
Fresh, unheated filtrate..	0.5	P2228	Spasms of the diaphragm; tremor and twitchings of the masseters and other muscles; recovery
Fresh, unheated filtrate, neutralized	1.0	R3507	Spasms of the diaphragm; tremor and twitchings of the masseters and other muscles; recovery
Fresh, unheated filtrate, neutralized	0.4	M 311	Tremor and twitchings of the masseters and muscles of the fore extremities; nystagmus; recovery
Fresh, unheated filtrate, treated with charcoal	1.3	R3635	No symptoms
Fresh, unheated filtrate, treated with charcoal	1.5	R3590	No symptoms
Fresh, unheated filtrate, dialyzed for 12 hours	1.7	R3637	Tremor and spasms of the masseters following two injections 7 hours apart; recovery
Fresh, unheated filtrate, dialyzed for 12 hours	1.6	R3638	Tremor and spasms of the masseters, muscles of the extremities and diaphragm following two injections 7 hours apart; recovery
Fresh filtrate heated to 60 C. for 1 hour	1.0	R3624	Tremor of the masseters
Fresh filtrate heated to 60 C. for 1 hour	1.0	R3532	Tremor of the masseters
Filtrate neutralized and in ice chest 24 hours	1.0	R3595	Marked tremor and twitchings of the masseters and muscles of the abdomen; spasms of the diaphragm; recovery
Filtrate neutralized and in ice chest 24 hours	1.4	R3634	Tremor of the masseters; recovery
Filtrate neutralized, in ice chest 24 hours and boiled 10 minutes	1.0	R3592	Slight tremor of the masseters; recovery
Filtrate neutralized, in ice chest 24 hours and boiled 10 minutes	1.5	R3535	Decided tremor of the masseters; recovery
Filtrate neutralized, in ice chest 24 hours and boiled 10 minutes	1.5	R3626	Decided tremor of the masseters; recovery
Filtrate neutralized, in ice chest 24 hours and boiled 10 minutes	2.0	R3636	Tremor and twitchings of the masseters; generalized muscular rigidity; marked lethargy; death
Unheated filtrate in ice chest 100 days	1.5	R3788	Marked tremor and spasms of the muscles of extremities; weakness; death
Unheated filtrate in ice chest 100 days	1.5	R3789	Marked tremor and spasms of the muscles of extremities; weakness; death
Filtrate of control incubated but uninoculated broth	1.5	R3610	No symptoms following 2 injections
Filtrate of control incubated but uninoculated broth	1.5	R3632	No symptoms following 2 injections
Filtrate of control incubated but uninoculated broth	1.0	R3633	No symptoms following 2 injections
Washed bacteria from 4 cc heated to 60 C. for 1 hour	0.4	R3548	Tremor and spasm of the masseters and muscles of the ears; recovery
Washed bacteria from 4 cc heated to 60 C. for 1 hour	0.5	R3639	Marked tremor of the masseters and twitchings of other muscles; recovery

TABLE 1—*Continued*

RESULTS ON INTRACEREBRAL INOCULATION OF THE LIVE CULTURE, THE BROTH CULTURE FILTRATE, AND DEAD BACTERIA OF STRAIN 4621

Material Injected	Dose Cc	Animal	Result
Washed bacteria from 4 cc heated to 60 C. for 1 hour	0.4	R3625	Spasms of the diaphragm; tremor and twitchings of the other muscles; death
Washed bacteria from 4 cc heated to 60 C. for 1 hour	0.7	R3491	Marked tremor of the masseters; twitchings of other muscles and spasms of the diaphragm; recovery
Washed bacteria from 4 cc heated to 60 C. for 1 hour and after in ice chest 100 days	0.5	R3790	No symptoms
Washed bacteria from 4 cc heated to 60 C. for 1 hour and after in ice chest 100 days	0.5	R3791	No symptoms
Washed bacteria killed with 1:1000 formalin	0.5	R3492	No symptoms
Washed bacteria killed with 1:1000 formalin	0.5	R3640	Marked spasms of the diaphragm; tremor and spasms of the masseters; death
Washed bacteria killed with 1:1000 formalin, and after in ice chest 100 days	0.5	R3793	Tremor and spasms of muscles of the abdominal wall and extremities; recovery
Washed bacteria killed with 1:1000 formalin, and after in ice chest 100 days	0.5	R3792	No symptoms
Bacteria after in dense glycerol-salt solution suspension 100 days	0.4	R3799	Marked tremor of the masseters; recovery
Bacteria after in dense glycerol-salt solution suspension 100 days	0.4	R3800	Marked tremor of the masseters; recovery
Bacteria in second culture and after in dense suspension of glycerol-salt solution 100 days	0.4	R3801	No symptoms

* The letters R, P, and M before the animal numbers indicate rabbit, guinea-pig, and monkey, respectively.

3635 and 3590. This substance or complex of substances is not dialyzable. Characteristic symptoms occurred in both rabbits injected (rabbits 3637 and 3638). Heating (60 C. for 1 hour and boiling for 10 minutes) diminished, but did not entirely destroy this property. Five of 6 rabbits injected developed tremor of the masseters but no spasms (rabbits 3624, 3532, 3592, 3535 and 3626), while the sixth (rabbit 3636) injected with the boiled filtrate developed marked lethargy, and tremor and twitchings of the masseters, and general muscular rigidity. The activity remained after preservation in the ice chest for 1 and 100 days, respectively. Marked tremor and twitchings or spasms of muscles occurred in 3 of 4 rabbits injected (rabbits 3595, 3788 and 3789). The fourth (rabbit 3634) developed tremor of the masseters only. The 3 rabbits (rabbits 3610, 3632 and 3633) injected as controls with the filtrate of the incubated but uninoculated broth, remained free from symptoms.

Of the 5 rabbits injected with the freshly washed, heat-killed bacteria (rabbits 3548, 3639, 3625 and 3790) (60 C. for one hour), 2 developed marked spasms of the diaphragm, and 2 tremor and twitchings of the masseters and other muscles. After preservation in dense suspension in salt solution in the ice chest, this property disappeared. The 2 rabbits injected remained free

from symptoms (rabbits 3790 and 3791). Of the 2 rabbits injected with the freshly washed and formalin-killed (1:1000) bacteria, 1 developed spasms of the diaphragm and other muscles (rabbit 3640), and the other remained free from symptoms (rabbit 3492). Likewise, 1 of the 2 injected with the same sized dose, after preservation in dense salt solution suspension in the ice chest for 100 days, remained free from symptoms (rabbit 3792), and the other developed tremor of the masseters and spasms of muscles of the abdominal wall and extremities (rabbit 3793). The 2 rabbits injected with the bacteria from this culture, preserved in dense glycerol-salt-solution suspension for 100 days, developed marked tremor of the masseters (rabbits 3799 and 3800), whereas the one injected with the same material from a subculture remained free from symptoms (rabbit 3801).

The second series consisted of 6 rabbits injected with material from the same cultures 24 hours later. Two were injected with the cleared centrifugalized broth; both developed marked spasms of muscles, including those of the diaphragm in one. The 2 receiving the freshly prepared filtrate remained free from symptoms after the first injection; 1 died of respiratory failure 3 hours after the second injection (given 7 hours after the first), and the other again remained free from symptoms. The two injected with the washed heat-killed bacteria (60 C. for 1 hour) developed marked tremor and twitchings of the masseters, and spasms of the muscles of the abdominal wall and the extremities.

In the third series, rabbits were injected with material from separate 72-hour glucose-brain-broth cultures of the 2 positive animals. The culture in the former had been layered with paraffin oil, that in the latter was incubated without oil. The filtrate of the former proved active in 2 rabbits injected; both developed tremor and twitchings of the masseters and 1 spasms of the diaphragm also. The filtrate of the latter was without effect in 4 rabbits inoculated. The dead washed bacteria produced marked spasms of the diaphragm, tremor and twitchings of muscles of the neck, extremities, abdomen and back in 2 rabbits injected, and also generalized convulsive attacks in one.

In the fourth series, I tested the effect of injections of 24-hour cultures from the fresh brain of one of the rabbits that developed spasms of the diaphragm after injection of the live bacteria in the mixture of the 72-hour cultures from the dried brain, from anaerobic glucose-broth cultures 3 months later, and from other old cultures. Six rabbits were injected with the cleared 24-hour broth cultures of the fresh brain, of the dried brain, and of the old anaerobic culture. Spasms of the diaphragm did not develop in any, but tremor and clonic spasms of other muscles occurred in 2 injected with cultures from the fresh brain. Two injected with the heat-killed bacteria from the anaerobic culture remained free from symptoms.

The filtrates, dead bacteria, and live streptococci from 24-hour glucose-brain-broth cultures of 3 additional old cultures, each isolated from the brain of positive animals, failed to produce tremor of the masseters or spasms of the diaphragm in any of 10 rabbits. One of these cultures had been kept for about 3 months on blood-agar slants, 1 in glucose-brain broth without oil, the other in glucose-brain broth layered with liquid paraffin. Each of these strains had retained its original cultural features, but the power of producing spasms of the diaphragm and other muscles, and tremor of the masseters, had disappeared.

The results in case 8 illustrate in general those obtained with the other 2 hiccup strains.²

The filtrates, dead bacteria, or live organisms of comparatively young cultures of the hiccup strains directly or not far removed from positive animals, were injected into 62 animals; spasms of the diaphragm occurred in 20. Thirty-four received filtrates; 9 developed spasms of the diaphragm as the only, or most striking symptom; 12 developed tremors and spasms of the masseters or other muscles, while 13 remained free from symptoms. Nineteen received dead bacteria; spasms of the diaphragm were noted as the striking symptom in 8; 6 had tremors or spasms of other muscles, and

TABLE 2

THE EFFECT OF INJECTION OF OLD MEAT INFUSION CULTURES OF HICCUP STRAINS (STRAINS 4573 AND 4602), CONTROL STRAINS (STRAINS 4564 AND 4605), AND OF THE UNINOCULATED MEAT INFUSION

Rabbit	Place of Injection	Amount, C c	Strain*	Result
3826	Brain	1.5	4573 ² .4	Marked tremor of the masseters; recovery
3827	Brain	1.0	4573 ² .4	Marked tremor of the masseters; weakness; death in 10 hours
3828	Brain	1.5	4564 ² .53 ⁵ .2	Respirations extremely marked, no tremors; death in 30 minutes
3829	Brain	1.0	4564 ² .53 ⁵ .2	Respirations extremely marked, no tremors; death in 10 hours
3830	Brain	1.5	4605 ² .3	No symptoms
3831	Brain	1.0	4605 ² .3	No symptoms
3813	Brain	1.5	4602.9 ² .2	Marked tremor of the masseters and other muscles; respiration moderately increased; death in 60 hours
3812	Brain	1.0	4602.9 ² .2	Marked tremor of the masseters and other muscles; respiration moderately increased; death in 60 hours
3837	Brain	0.5	4602.9 ² .2	Marked tremor of the masseters; respiration slightly increased; recovery
3838	Brain	0.05	4602.9 ² .2	Decided tremor of the masseters; respiration normal; recovery
3839	Brain	0.005	4602.9 ² .2	Moderate tremor of the masseters; respiration normal; recovery
3834	Ear vein	8.0	4602.9 ² .2	Moderate tremor of the masseters; respiration normal; recovery
3836	Ear vein	2.0	4602.9 ² .2	Slight tremor of the masseters; respiration normal; recovery
3835	Ear vein	8.0	4564 ² .53 ⁵ .2	No tremor of the masseters or other muscles; respiration moderately increased
3832	Brain	1.5	Control meat infusion	No symptoms
3833	Brain	1.0	Control meat infusion	No symptoms
3822	Brain	1.0	Control meat infusion	No symptoms
3823	Brain	1.5	Control meat infusion	No symptoms

* The exponent to the right and above the numbers of the strain indicates the animal passage; the figure following the point, the culture generation.

5 remained free from symptoms. Eight were injected with live organisms; 3 developed spasms of the diaphragm; 3 tremor or spasms of other muscles, and 2 remained free from symptoms. Thirty-three were injected with filtrates, dead bacteria, and live cultures of the same strains after cultivation on artificial mediums other than meat infusion for some time, up to 3½ months; 21 received filtrates, 4 dead bacteria, and 8 live cultures. None of these developed spasms of the diaphragm and only 4 had tremors or spasms of other muscles. Seven animals were injected with the filtered but uninoculated broth as controls; all remained free from symptoms.

Cultures from the blood and brain of animals injected with sterile filtrates and suspensions of dead bacteria, chloroformed for examination, or dying from the effects of the injection, did not yield the streptococcus. In order to rule out all possibility that the symptoms were due to the streptococcus or substances produced by it and not to a filtrable and heat resisting virus, emulsions of the brains of 4 rabbits with positive symptoms following inoculation of filtrates and heat-killed bacteria were inoculated directly into the brain of 4 normal rabbits; all remained free from symptoms.

The respiratory arrhythmic strain (strain 4564) in similar experiments produced marked hyperpnea but no spasms of muscles. It retained this characteristic property after long cultivation in meat infusion (table 2).

The freshly isolated cultures from the tonsils and spinal fluid in the case of encephalitis (strain 4605) produced convulsions in a series of rabbits as the striking symptom. The filtrate of the 24-hour culture in glucose-brain broth of the streptococcus from the spinal fluid in the second culture produced clonic spasms of muscles of the extremities, and of the back and abdomen in 1 rabbit injected with 1 cc and clonic spasms of the muscles of the hind extremities in 1 rabbit receiving 0.5 cc.

The filtrate of an identical culture from the tonsil was without effect in the 2 rabbits injected. The culture from the brain of 1 rabbit inoculated with the live culture from the spinal fluid in the second subculture was inoculated into the meat-infusion medium. After 3 months, it had lost the characteristic power of producing spasms of muscles (table 2).


PROTOCOLS OF EXPERIMENTS

Rabbit 3507, weighing 2,000 gm., was injected intracerebrally, March 17, at 3:30 p. m., with 0.5 cc of the filtrate of a heated, neutralized 24-hour glucose-brain-broth culture of the strain from the tonsil in case 7 in the tenth rapidly made subculture.² The temperature was 102, and the leukocytes numbered 12,000. At 8 p. m., the respirations were moderately increased, and the animal had developed repeated, irregular spasms of the diaphragm of varying intensity (fig. 1). At 8:30 p. m., the spasms had become more marked and at times violent, but the severity and the intervals between the spasms varied greatly (fig. 1). The temperature was 106.2; the animal repeatedly attempted to control the spasms by crouching to the floor of the cage. At 10:30 p. m., the spasms of the diaphragm continued but were less marked, and the animal was more comfortable. March 18, at 7 a. m., the animal appeared well; there was an occasional slight spasm of the diaphragm, the respirations were somewhat irregular (fig. 1), the temperature was 103, and the leukocytes numbered 14,400. The animal remained well, and on March 20, another respiratory tracing (fig. 1) was taken, which showed normal respirations. On March 22, the injection was repeated, but symptoms did not develop.


March 27, at 3 p. m., 0.5 cc of this filtrate was again injected intracerebrally. At 5 p. m., respirations were markedly increased. The animal sat quietly, appeared uncomfortable, and disinclined to hop; when it was made to do so, irregular clonic spasms of the diaphragm occurred. At 9 p. m., respirations were still moderately increased, and the animal had repeated spasms of the diaphragm. March 28, at 8 a. m., the animal appeared well. April 10, at 4 p. m., it received 1 cc of the fresh unheated filtrate of the neutralized glucose-brain-broth and ascites-glucose-broth cultures of the brain of 2 rabbits that had spasms of the diaphragm following injection of the filtrate of the streptococcus from case 8.² At 4:30 p. m., respirations

were decidedly increased, the animal appeared afraid to move and when it did so, repeated clonic spasms of the diaphragm, tremors, and spasms of the muscles of the ears were noted. The spasms of the diaphragm were repeatedly controlled by pressure of the hands around the abdomen, but they recurred promptly on release of the pressure. At 6 p. m., spasms of the diaphragm and masseters had ceased. At 10 p. m., respirations were much increased, but there were no spasms. April 11, at 2 a. m., the animal appeared quite well, and there were no spasms of the muscles, or tremors. April 12, the animal appeared definitely sleepy, responded slowly to prodding, and held its ears forward and erect. April 13, it was still somewhat slow


march 20 11 a.m.



march, 18 7 a.m.




march, 17, 8:30 P.m.



march 17. 8 P.m.



march, 17, 1922 11:05 a.m.



Time in seconds



Respiratory record Rabbit 3507 Injected march 17, 3:30 P.m.

Fig. 1.—Respiratory records of rabbit 3507, showing spasms of the diaphragm.

and sleepy. April 14, it appeared well, and remained so until Aug. 3 when, at 3:05 p. m., it was injected with 1 c c of a mixture of the old meat-infusion culture under oil of strains 4602 and 4573. At 4 p. m., there were decided tremors of the masseters; the animal was not observed until the following morning at 7 a. m., when it was found dead; the body was warm.

Examination revealed marked digestion of the stomach and perforation of the diaphragm from gastric digestion, turbid cerebrospinal fluid, moderate edema of the lungs, and no lesions of the heart valves. The cultures from the blood remained sterile; those from the brain yielded a pure growth of the characteristic streptococcus.

Rabbit 3549 was injected intracerebrally March 22, at 11:25 a. m., with 0.5 c c of the heated (60 C. for 1 hour) neutralized, clear, centrifugalized

glucose-brain-broth culture. The broth had been inoculated 24 hours previously with a mixture of a culture of strain 4602 in the ninth and tenth subcultures and after 1 animal passage. This suspension had been kept in the ice-chest for 1 week. At 4 p. m., the animal appeared well; at 4:30 p. m., it appeared uncomfortable, was afraid to move and often crouched flat to the floor of the cage; at 5 p. m., the respirations had increased, it was highly irritable, the slightest tap over the back provoked spasms of the muscles, and, after hopping, clonic spasms of the diaphragm developed. At 9:30 p. m., respirations were less marked; the animal appeared more comfortable, and the muscular spasms had disappeared. At 7:30 a. m., it appeared well, respirations were normal, and spasms were absent. March 27, it was injected

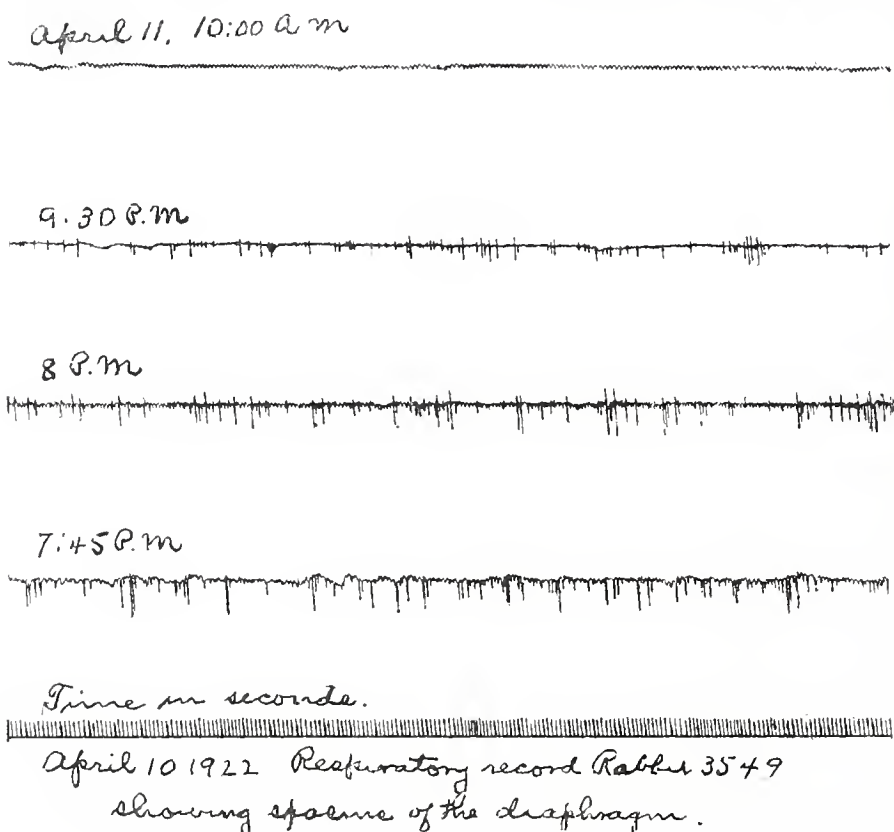
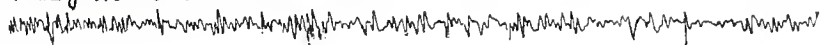


Fig. 2.—Respiratory records of rabbit 3549, showing spasms of the diaphragm.

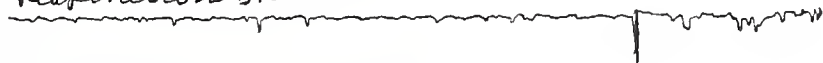
with 0.5 c.c. of a filtrate of the heated culture. No spasms of the muscles followed this injection, and the animal again remained well. April 10, at 4:05 p. m., it was again injected with 1 c.c. of an active filtrate from case 8.² At 5 p. m., there were marked tremor and twitchings of the masseters, and muscles of the neck. At 7 p. m., tremor and twitchings of the masseters ceased when the animal was at rest, but when placed under tension distinct tremors appeared. At 7:45 p. m., marked spasms of the diaphragm of varying intensity and rate developed (fig. 2). At 8 p. m., spasms continued unabated (fig. 2). At 9:30 p. m., the diaphragmatic spasms were still present, but were less marked and occurred more irregularly (fig. 2); at 11 p. m., the

spasms were slight. April 11, 2 a. m., the spasms of the diaphragm were absent; at 10 a. m., the animal appeared quite well, and hopped. There were no tremors or spasms of the masseters unless under tension, when paroxysms of fine tremor developed. The respirations were normal (fig. 2). At 8:30 p. m., tremors of the masseters had disappeared, and the animal remained well for many weeks while under observation.

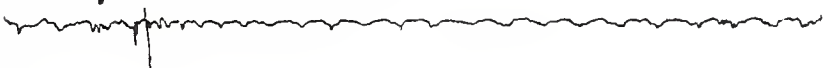
Respiration 10:00 P.M.



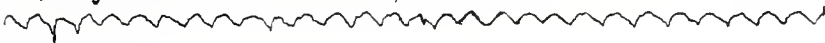
Respiration 8:30 P.M.



Respiration 8:15 P.M.



Respiration 4:00 P.M.



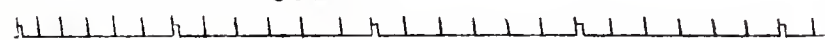
Right masseter 4:00 P.M.



Left masseter 4:00 P.M.



Time in seconds



*April 10 1922 Record of respirations showing
spasms of diaphragm and abdominal muscles
and of masseter muscles Rabbit 3623.*

Fig. 3.—Respiratory records, showing spasms of the diaphragm, abdominal muscles, and of the masseter muscles in rabbit 3623.

Rabbit 3623, weighing 1,850 gm., was injected intracerebrally, April 10, at 3 p. m., with 1 cc of the filtrate of the mixture of equal parts of 24-hour cultures in glucose-brain broth and ascites-glucose broth inoculated with the brain emulsions of 2 rabbits that had spasms of the diaphragm (case 8).² At 3:20 p. m., there were marked spasms of the masseters, but none of the

diaphragm. At 4 p. m., tremors and spasms of the masseters continued; they varied greatly in number on the 2 sides and occurred at irregular intervals. The paroxysms of the spasms usually occurred simultaneously on both sides, but the individual spasms were much more numerous on the left than on the right side. There were intervals when the right masseters beat the time, as it were, while spasms of the left side occurred in rapid succession, resembling fibrillations. Tracings were taken simultaneously of the 2 sides (fig. 3). The respirations at the same time were somewhat irregular and the abdominal muscles twitched. The twitching is shown as slight waves in the respiratory record (fig. 3). There were also sharp twitchings of the muscles of both ears, and the neck and shoulders. At 8:15 and 8:30 p. m., the tremors and spasms of the masseters had disappeared, but spasms of the muscles of the neck, shoulders, back, and abdomen were more marked, and there was an occasional sharp spasm of the diaphragm (fig. 3). At 10 p. m., the spasms of the muscles of the neck, shoulders, back and abdomen had practically ceased, but relatively slight spasms of the diaphragm occurred with almost every respiratory cycle (fig. 3). At 11 p. m., the animal was more comfort-

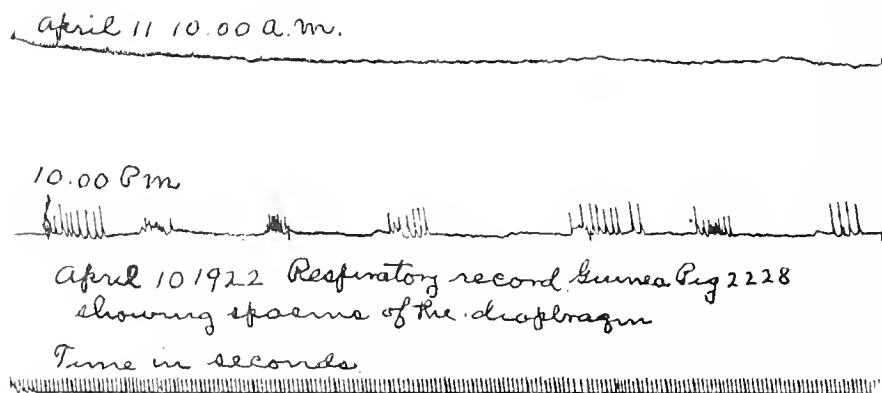


Fig. 4.—Respiratory records of guinea-pig 2228, showing spasms of the diaphragm, and recovery.

able; the spasms of all muscles had largely disappeared, but there was still an occasional sharp spasm of the diaphragm.

April 11, at 2 a. m., the animal appeared well, was alert, hopped normally, and tremors and spasms of the muscles had disappeared. At 8 a. m., the masseters did not tremble when the animal was quiet, but on an attempt to pry open the lower jaw, decided tremor developed. At 3:45 p. m., the animal was reinjected; at 4:45 p. m., there was marked tremor of the masseters and muscles of the ears, neck, shoulders and back. At 6 p. m., the condition was much the same; at 8:30 p. m., spasms of the muscles of the ears, neck and shoulders had disappeared, but tremors of the masseters were still present when under tension.

April 12, at 10:15 a. m., tremors and spasms of the muscles had disappeared, and the animal had grown sleepy. It did not respond to slight stimulation, and response to more severe stimulation was delayed.

April 13, at 8:30 a. m., the animal sat quietly in the cage apparently half asleep, and breathed slowly without tremor or twitchings of the muscles. There was moderate rigidity of the muscles of the extremities, and the animal tended to lose its balance, falling usually to the left. At 4 p. m., the con-

dition was about the same. When taken to the photographic room for a motion picture, the animal was awakened and developed a coarse tremor of the muscles of the fore part of the body, which disappeared as it again fell asleep with eyes half closed, and ears pointing forward; at 6 p. m., it could not be aroused to eat carrot. On April 14, the animal was still lethargic, but awakened sufficiently to eat when food was placed in its mouth. On April 15, it appeared brighter, was more active, could be aroused readily and ate oats and hay with relish. It recovered completely, and no further symptoms developed during the course of 3 weeks.

Guinea-pig 2228, weighing 325 gm., was injected intracerebrally April 10, at 5:45 p. m., with 5 cc of the filtrate injected into rabbit 3623. At 7 p. m.,

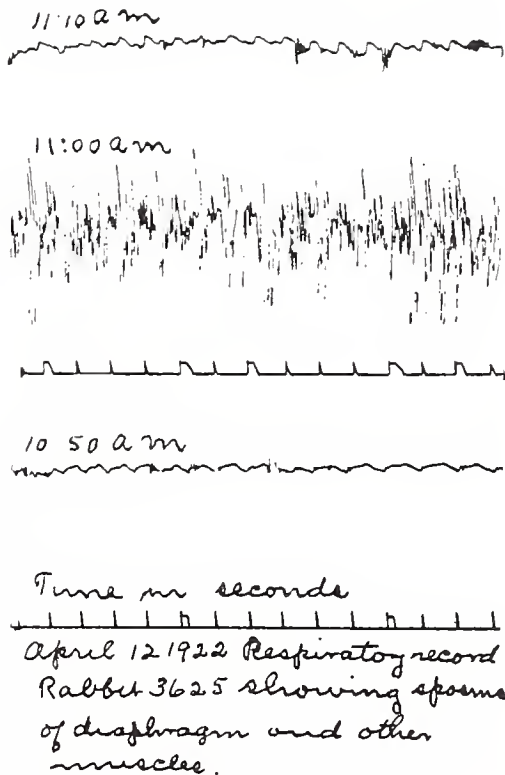


Fig. 5.—Respiratory record of rabbit 3625, showing spasms of the diaphragm and other muscles.

no spasms of the muscles occurred. At 8:30 p. m., the muscles of the ears and eyelids twitched repeatedly, and respirations were rapid. At 9:30 p. m., the animal had developed violent spasms of the abdominal muscles and diaphragm. With the more severe spasms of the diaphragm, audible hiccup occurred repeatedly. At 10 p. m., the spasms of the abdominal muscles had grown less marked, but were numerous. They are shown in the respiratory tracing as fine oscillations (fig. 4). Spasms of the diaphragm were more marked and occurred almost wholly in paroxysms of from 4 to 9 spasms, usually at the rate of about one each second in each paroxysm, but occasional showers of spasms resembling fibrillations occurred (fig. 4). At

11 p. m., the spasms of the diaphragm continued, but tremor and twitching of the abdominal muscles had disappeared. At 2:40 a. m., the animal was more comfortable; there were occasional twitchings of the muscles of the eyelids and ears, and only a few mild spasms of the diaphragm. At 10 a. m., there were no spasms of the muscles, and the animal remained well.

Rabbit 3625, weighing 1,740 gm., was injected intracerebrally April 10, 1922, at 3:30 p. m., with washed bacteria from 4 cc of a 24-hour neutralized, heated (60 C. for 1 hour) glucose-brain-broth and ascites-glucose-broth culture inoculated with the brain emulsions of 2 rabbits that had spasms of the diaphragm (case 8).² At 4:30 p. m., the animal appeared well; there were no spasms and no tremors of the masseters. At 4:45 p. m., there was fine tremor with occasional slight spasms of the masseters; at 6 p. m., the spasms and tremors of the masseter muscles continued, and at 10 p. m., the animal was also somewhat ataxic. April 11, at 2 a. m., respirations were much increased, the animal held its head to the right and backward, and when prodded, ataxia became evident; tremor and occasional twitching of the masseters continued. At 8 a. m., and at 8:45 p. m., the condition was unchanged. April 12, at 10:50 a. m., the animal was found sitting quietly

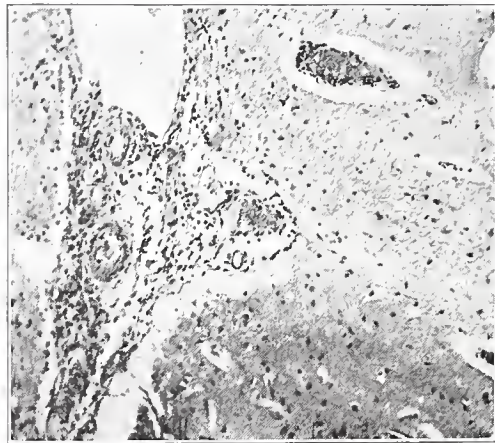


Fig. 6.—Leukocytic infiltration of the pia of the sulcus, and perivascular infiltration near the base of the cerebrum of rabbit 3625. Hematoxylin and eosin; $\times 100$.

in the cage, apparently uncomfortable on account of sharp twitchings of the muscles of the back, abdominal wall and thighs. The spasms of the abdominal muscles appeared as slight oscillations in the respiratory tracing (fig. 5). At 11 a. m., the animal had developed violent clonic spasms of the diaphragm associated with a frequent audible inrush of air through the glottis (fig. 5). At 11:10 a. m., the clonic spasms of the abdominal muscles continued, respirations were irregular, and occasional showers of spasms of the diaphragm, not unlike fibrillations of this muscle, were noted (fig. 5). At 12:45 p. m., the spasms of the diaphragm had disappeared, but the animal developed, at intervals, sharp tremors and twitchings of most of the muscles of the entire body. At 9 p. m., the condition was much the same, but tremors and twitchings of the muscles had almost disappeared. The animal was weak and very ataxic. At 4:13 p. m., it was found dead.

Necropsy revealed extreme congestion of the vessels of the meninges and edema of the pia over the anterior aspect of the pons and medulla and over the posterior surface of the cerebellum. Cultures from the brain and blood remained sterile.

Sections showed marked leukocytic infiltration in both lateral ventricles surrounding the choroid plexus, slight leukocytic and perivascular infiltration in the pons and lower subcortical region of the cerebrum on both sides, moderate leukocytic infiltration surrounding the vessels in the sulci over the cerebrum, more marked near the base (fig. 6); leukocytic infiltration of the pia over the anterior aspect of the pons and posterior portion of the cerebellum, and a large hemorrhagic area in the posterior aspect of the cerebellum. Diplococci, chiefly within leukocytes, were readily demonstrable in the lesions (fig. 7). These showed marked evidence of disintegration, some were very large and swollen, others of normal size, deeply stained; many appeared as granules of varying sizes within leukocytes.

Rabbit 3631, weighing 1,490 gm., was injected April 11, at 3:05 p. m., with 1 cc of clear supernatant broth of a 48-hour culture in glucose-brain broth and ascites-glucose broth of the 2 positive rabbits inoculated, 1 with salt solution suspension from the nasopharynx, and the other with the primary glucose-brain-broth culture of strain 4602. At 4:30 p. m., it appeared well; tremors and spasms of the muscles were absent. On April 12, at

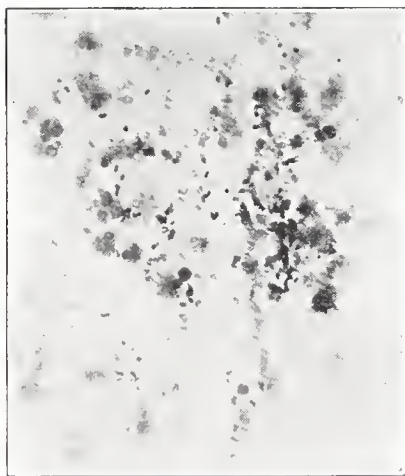


Fig. 7.—Diplococci in area of leukocytic infiltration in the pia. Gram-Weigert; $\times 1000$.

10:05 a. m., respirations were somewhat increased, the animal was excitable and held its body very rigid, but there were no spasms or tremors of the muscles. At 2:45 p. m., the condition was unchanged. At 9 p. m., tremors of the fore part of the body, ataxia, and slight turning of the head had developed. April 13, at 8:30 a. m., the animal was found sitting in a prone position in the cage, apparently attempting to control frequently repeated rhythmic clonic spasms of the diaphragm and muscles of the abdominal wall. At 10:05 a. m., spasms of the diaphragm were extremely marked and occurred with almost every respiratory excursion (fig. 8). With the severe spasms, the animal winced repeatedly, and when it was prodded sharp spasms of the muscles of the extremities developed. At 2:50 p. m., the spasms of the diaphragm continued, but were less violent. At 5:15 p. m., the condition was about the same, but the animal had developed violent paroxysmal spasms of the diaphragm, during which it became excited and had great difficulty

in breathing (fig. 8). At 7:35 p. m., spasms of the diaphragm continued, the paroxysmal attacks became more numerous, and as the tracing was taken (fig. 8), the spasms suddenly disappeared. The diaphragm fibrillated for a few seconds, followed by 2 violent respirations as death occurred. These recurring attacks of violent spasms with embarrassed respirations were also noted in the rabbit inoculated directly with the salt solution suspension from the throat of the patient in this case (case 7).²

Necropsy revealed moderate cloudiness and edema of the pia over the medulla and cerebellum; there was no mark at the point of injection and no gross hemorrhage. The phrenic nerve and the diaphragm were free from lesions; cultures in glucose-brain broth from the brain yielded the characteristic streptococcus; those from the blood were sterile.

Microscopically, there was leukocytic and round-cell infiltration of the pia, which was slight over the cerebral cortex, more marked over the lower rolandic region and pons, and most marked over the anterior aspect of the medulla,

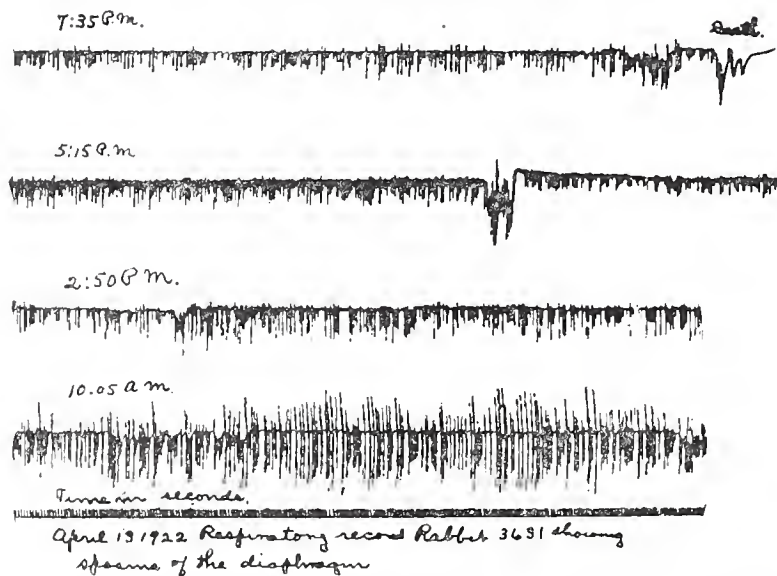


Fig. 8.—Respiratory records of rabbit 3631, showing spasms of the diaphragm.

edema, hemorrhage within and without, and leukocytic infiltration surrounding blood vessels in the anterior part of the medulla and pons. Large numbers of gram-staining diplococci and short chains were found in the areas of perivascular infiltration, but not elsewhere.

Rabbit 3636, weighing 1200 gm., was injected intracerebrally, April 11, at 6 p. m., with 2 cc of a filtrate of the 24-hour culture of the 2 positive rabbits, injected with the strain from case 8,² after the culture had been kept in the ice-chest during the night and then boiled for 10 minutes. At 7 p. m., the animal sat quietly with ears erect; it appeared rather weak, and moved slowly; respirations were normal. There was tremor of the masseters when they were under tension. At 8 p. m., the condition was unchanged.

April 12, at 10 a. m., the animal sat quietly in the cage apparently asleep, but had repeated coarse tremors and sharp twitchings of the masseters on the left side and slighter twitchings on the right side. When prodded it

awakened slowly, and the spasms of the masseters disappeared, but in a few moments it again fell into deep sleep. At 1 p. m., it sat apparently asleep with ears held rigid, and did not respond to moderate stimulation, but it moved slowly and stiffly from one place to another. The tonus of the muscles of the extremities was increased, and when the animal hopped, the whole body tended to fall in a mass. At 5 p. m., it appeared sound asleep and held its jaws rigid. There was continuous fine tremor of the masseters, which was made worse when they were under tension. It gave little resistance when placed on its side and back with the extremities in the air. When the animal was thoroughly aroused, it hopped stiffly, often with swaying body, until it nearly fell over. At 9 p. m., it was found lying on its side apparently asleep, and when watched for a long period, it would, of its own accord, get on its feet, walk slowly, and raise itself high in the air. At 11 p. m., its actions were unchanged. On April 13, 8:30 a. m., the animal was found lying on the left side, apparently sound asleep. When it stood up and attempted to hop, it lost its balance. After each exertion, it fell into deep sleep. The tonus of the muscles of the extremities was markedly increased,

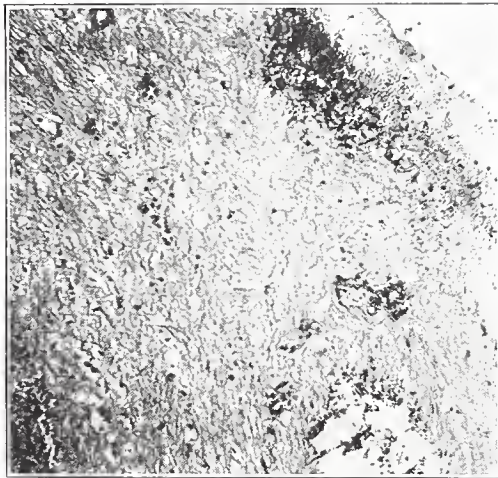


Fig. 9.—Areas of hemorrhage and necrosis in upper and anterior part of the pons of rabbit 3636. Hematoxylin and eosin; $\times 100$.

and when the animal was placed on its back and the extremities held in a rigid extended position, it continued to sleep. At 4 p. m., the condition was unchanged. A motion picture was taken. The animal could not be aroused sufficiently to eat pieces of cabbage placed in its mouth. At 10 p. m., it was extremely sleepy, the respirations were slow, and it was aroused with great difficulty. After prolonged prodding, it awakened sufficiently to get on its feet, but promptly drooped its head, closed its eyes, and fell slowly to the right with extremities in an extended position. On April 14, at 7 a. m., the animal was found dead; the body was warm.

Necropsy revealed the cerebrospinal fluid to be clear but increased in amount, a few hemorrhages in the pia over the middle and anterior aspect of the medulla on the right side, but no other gross lesions. Cultures from the blood and brain remained sterile.

The microscopic lesions consisted of large and small areas of necrosis and hemorrhage with little or no cellular infiltration near the base of the

cerebrum, anterior part of the pons (fig. 9) and in the medulla and cerebellum, and slight mononuclear and leukocytic infiltration around the blood vessels of the pia over the anterior aspect of the pons and medulla. There were no lesions in the choroid plexus, and no bacteria in the areas of necrosis. The areas of hemorrhage and necrosis were generally so large that they could easily be recognized with the naked eye in the sections of the cerebrum corresponding to the corpus striatum and surrounding structures.

GROSS AND MICROSCOPIC LESIONS

Necropsy in most instances revealed only slight changes in the central nervous system, and no abnormalities of the other viscera. Congestion of the vessels of the meninges of varying degree, always more marked over the base of the brain, pons, medulla and cervical cord, was an almost constant finding. The cerebrospinal fluid was usually slightly or moderately turbid, the degree of turbidity varying with the duration of the experiment. It was most marked in animals that died within 24 to 72 hours following injection

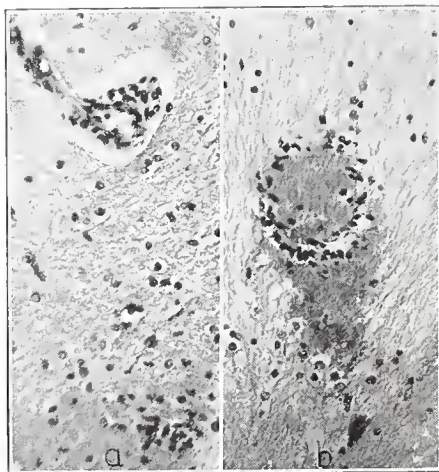


Fig. 10.—Localized and perivascular hemorrhage, and leukocytic infiltration in the brain of rabbits inoculated 24 hours previously, *a*, with an active filtrate, and *b*, with dead bacteria. Hematoxylin and eosin; $\times 100$.

of live organisms, less pronounced after injection of the dead streptococci, and least marked following injection of filtrates. Gross hemorrhages at the point of inoculation or elsewhere were rare; occasionally, these were found in the pia along the anterior cervical nerve roots, anterior aspect of the medulla, and in the cerebellum. A circumscribed area of edema and infiltration of the pia over the anterior and upper part of the medulla was found in nearly all animals that died in from 24 to 72 hours after inoculation of the dead or live streptococci.

Smears from the turbid fluid pipetted from these areas showed a variable number of leukocytes, large and small mononuclear cells, the proportion depending on the duration of the experiment, and a variable number of diplococci. The diplococci were usually situated within cells, were most numerous and of quite normal size and shape in the animals injected with live streptococci, and less numerous and of extremely variable size and shape in those injected

with dead streptococci. Some were extremely large, usually outside of leukocytes, while others were extremely small and within leukocytes or large mononuclear cells.

Sections of the cerebrum, pons, cerebellum, medulla and upper cervical cord were studied in 51 animals injected with material from cases 7 and 8.² Twenty-five of these were injected with live cultures of strains freshly isolated, after from 1 to 3 animal passages and relatively soon after artificial cultivation. Of these, 3 showed no symptoms, 8 developed spasms of the diaphragm, 2 spasms of the abdominal muscles and 11 of other muscles. Twenty developed twitchings or spasms of muscles as the chief symptom, 2 nystagmus, 2 tic-like movements of the head, 6 tremors of the masseters, 7 hyperpnea of varying degree, 3 ataxia, and 1 lethargy. Fifteen were injected with dead bacteria. Of these, 1 showed no symptoms, 7 developed spasms of the diaphragm, 3 of abdominal muscles, and 8 of other muscles. In all, 12 developed spasms of muscles as the chief symptom, 3 tremors of the masseters, 2 ataxia, 1 paralysis, 1 lethargy, and 8 hyperpnea of varying degree.

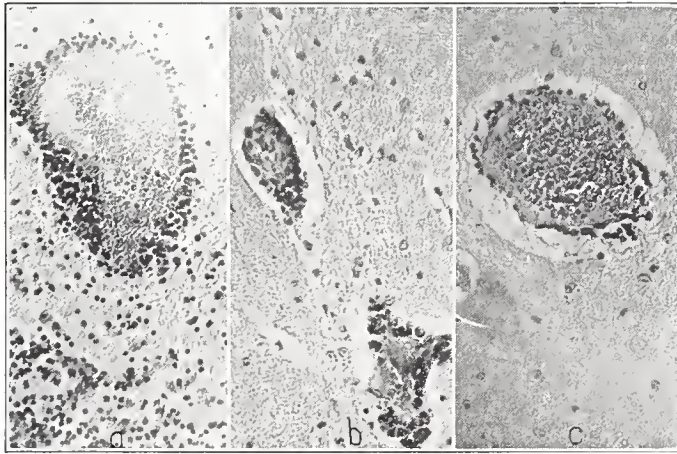


Fig. 11.—*a*, Marked localized and perivascular round-cell infiltration in the pons of a rabbit inoculated 16 days previously with a live culture in the tenth subculture; *b*, moderate perivascular round-cell infiltration in rabbit chloroformed 3 days after the third, and 13 days after the first intracerebral inoculation of the heat-killed streptococci; *c*, slight perivascular round-cell infiltration in the pons of a rabbit 3 days after 2 daily intracerebral injections of the filtrate of strain 4602.

Eleven were injected with filtrates; of these, 2 remained free from symptoms, 2 developed spasms of the diaphragm, 1 of the abdominal muscles, 4 of other muscles, 7 muscular spasms of some degree, 3 tremor of the masseters, 2 hyperpnea, and 2 lethargy. One of the 2 that became lethargic was injected once (rabbit 3636), the other twice, 24 hours apart.

The microscopic changes in these 3 groups of animals were similar in kind and location, but varied greatly in extent and degree, depending on the duration of the experiment, the number of injections, and the material injected. The changes were most widespread and most marked following inoculation of the live organisms, less extensive and less intense after injection of the dead streptococci, and least marked after injection of the filtrates.

The lesions, soon after inoculation, consisted in the main of moderate leukocytic infiltration of the pia, particularly at the base and around the

blood vessels (fig. 10a and b); localized and perivascular hemorrhage (fig. 10b), and leukocytic infiltration was usually most marked in the lower and deeper layers of the cerebrum, and in the pons and anterior part of the medulla. Demonstration of diplococci, often in short chains in these areas, was readily accomplished in the animals injected with dead (figs. 6 and 7) and living organisms, but was not possible in those injected with filtrates. In rare instances, large areas of hemorrhage and necrosis, with little cellular reaction, were found (fig. 9). The lumina of the vessels were nearly always free from accumulations of cells, and the endothelial lining was intact, even when marked perivascular infiltration by leukocytes and bacteria had occurred. Occasionally, endothelial necrosis was found, usually in vessels immediately adjacent to areas of hemorrhage or necrosis and marked leukocytic infiltration (fig. 10b).

As the duration of the experiment increased, a progressively larger proportion of mononuclear cells appeared, the location and degree of which paralleled that of the earlier leukocytic infiltrations. It was most marked after injection of the live streptococci, less marked after injection of the dead organisms, and least marked on introduction of filtrates (fig. 11a, b and c).

Perivascular and localized hemorrhages in the medulla and pons, especially after repeated injections of filtrates and dead bacteria, sometimes appeared to be the cause of death in animals that were seemingly well. Infiltration of anterior cervical nerve roots was found after inoculation of live cultures, dead streptococci and filtrates.

Animals that lived for a week or 10 days after a single injection of the filtrate usually showed no microscopic changes. Those injected once with the dead bacteria showed mild round-cell infiltration, while those injected repeatedly with the dead bacteria usually had marked perivascular and localized infiltration by mononuclear cells. None of the 27 rabbits that died or was chloroformed soon after inoculation of filtrate, dead bacteria or live culture, revealed mononuclear perivascular infiltration. The 24 that were examined after inoculation showed a varying degree of leukocytic and round-cell infiltration, corresponding precisely to the duration of the experiment and the nature of the material injected. The symptoms and microscopic lesions in a number of guinea-pigs paralleled those in the rabbits. No lesions were found in the sections of 14 rabbits inoculated with inert filtrates and brain emulsion from the animals injected with freshly isolated strains from 8 cases of epidemic hiccup.

EXPERIMENTS ON THE EFFECTS OF OLD MEAT-INFUSION CULTURES

Cultures from the brain of positive rabbits made about the same time and inoculated into bottles containing 120 c.c. of the meat-infusion medium, were available for comparative study about 3½ months later. These, with the uninoculated meat infusion, also layered with oil, had been incubated for 24 hours, and were then kept at room temperature in the dark. Two of the strains were (cases 4 and 7)² from patients with epidemic hiccup, one (case 4564) from the case of respiratory arrhythmia and one (case 4605) from acute encephalitis with convulsions.

Blood-agar plates yielded a pure culture of the streptococcus in approximately the same number for each strain. All of the strains produced similar green colonies as when first isolated. The immediate effect of the soluble substances and the later effect of the live organisms were determined by injecting the usual amount of the slightly turbid, opalescent, old culture of each intracerebrally into animals, and the uninoculated meat infusion as

controls. The effect of one of the hiccup strains and the respiratory arrhythmic strain was also studied on intravenous injection and in high dilution on intracerebral inoculation. From the results summarized in table 2, it is clear that 3 of the 4 strains studied produced distinctive poisons in this medium. Although the hiccup cultures lost the power of producing spasms of the diaphragm, they produced marked tremor of the masseters (rabbits 3826, 3827, 3812, 3813, 3837 and 3838). This effect in one strain (case 7)² was so pronounced that even 0.005 cc injected into the brain (rabbit 3839) sufficed to produce the characteristic reaction, and what is still more significant, larger doses injected intravenously (rabbits 3834 and 3836) had precisely the same effect. The respiratory arrhythmia strain (case 4564), however, produced marked hyperpnea but no tremors on intracerebral injection, and moderately increased respiration on intravenous injection (rabbits 3828, 3829 and 3835). The encephalitis strain (case 4605) had no apparent effect (rabbits 3830 and 3831). The control animals injected with the uninoculated meat infusion remained free from these symptoms (rabbits 3822, 3823, 3832 and 3833). A few of the animals had spells soon after injection, during which they shook their heads and scratched their noses, the sides of the head and ears severely for a few minutes. This was quite independent of the other symptoms, and was as common and as severe in the controls as in those injected with the cultures.

In a subsequent series of experiments with a mixture of the old hiccup cultures, and with the respiratory arrhythmia culture, results similar to those recorded in table 2 were again obtained. Stimulated by the recent report of Bronfenbrenner concerning the extraordinary power of the toxin of *Bacillus botulinus* in high dilution, the effect of dilutions in sodium chlorid solution of the active filtrate of the mixture of the hiccup strains was studied. The dilutions were made immediately before injection. One-half cc of the undiluted filtrate was injected, and the result noted. If tremor of the masseters developed within 15 minutes, the next animal was injected with 0.5 cc of a 1:10 dilution, and subsequent cultures were increasingly diluted until no symptoms were obtained. These experiments were controlled by injecting the same amount of sodium chlorid solution used in the making of the dilutions. Each of the 4 rabbits injected with dilutions up to 1:1000 developed marked tremor of the masseters; the 2 injected with the 1:1000 dilution developed moderate tremor of these muscles; the 4 injected with dilutions of 1:100,000 to 1:100,000,000 developed decided tremor of the masseters, and the one injected with the 1:1,000,000,000 dilution remained free from symptoms. Of the 9 injected with the sodium chlorid solution, 4 developed slight tremor of the masseters, and 5 remained free from symptoms. It was found with this filtrate, as with those from freshly isolated cultures, that animal charcoal removed the spasm-producing substance or complex.

In an experiment I tried to separate the spasm-producing substance by precipitation with alcohol, but without success. The amount of precipitate obtained by adding equal parts of 95% alcohol was fully twice as great in the filtrate of the old culture as in the filtrate of the uninoculated meat infusion. The dried precipitate of each dissolved in an amount of sodium chlorid solution equivalent to the filtrate, and injected intracerebrally in the usual dose was without effect in 4 rabbits. It was thought that since this tremor-producing property was present in these old cultures, it would remain active in the filtrate indefinitely. However, it disappeared more quickly in this filtrate than in filtrates from freshly isolated, active cultures producing spasms of the diaphragm. When the filtrates were kept at room

temperature on a hot day for 6 hours, while a series of rabbits were being injected, progressively diminishing effects were noted, and the following day, even though kept on ice, 1 filtrate was without effect in 2 rabbits and produced slight tremor of the masseters in 1 other.

The effect of the mixture of the old meat-infusion cultures of the hiccup strains was studied in 12 rabbits previously injected intracerebrally from 2 to 4 times, over a period of 6 weeks, with active filtrates, dead bacteria and live cultures. The last previous injection was given at least 10 days before. All of these developed tremor, with or without twitchings of masseters, comparable in time of onset, severity, and duration with those of the previously uninjected rabbits. Five of these rabbits had had spasms of the diaphragm and tremor of the masseters following 1 or 2 previous injections; 5 others had tremor and twitchings of the masseters or other muscles following previous injections, while 2 remained free from symptoms following each previous injection. Moreover, these animals were as susceptible as normal rabbits to infection by the relatively few live streptococci contained in these old cultures.

IMMUNIZATION EXPERIMENTS

Since the effect of the poison in these filtrates and dead bacteria was so specific and so readily determined objectively, it was thought worth while to attempt to immunize animals with the filtrates and the dead bacteria, respectively. Fifty-six rabbits received 2 inoculations, 15 received 3, 5 received 4, and 1 received 5 inoculations of filtrates, dead bacteria, or live cultures. Seventeen were inoculated repeatedly with filtrates of the hiccup strains only. Marked evidence of the development of immunity to 1 or more injections occurred in 6, while in 11 no increased resistance was apparent. In each instance in which immunity was noted, the filtrate in the test injection was the same as the previous strain, and was known to be active for normal rabbits at the time of injection. Three of the 6 immune animals were found to be susceptible to a subsequent injection of a filtrate of a different hiccup strain (protocol of rabbit 3549). Eleven received repeated inoculations of dead bacteria only. Three of these showed evidence of immunity, while 8 did not. In all of the former the test injection was the same as the original. Eighteen received filtrates, dead bacteria, and live cultures, chiefly from epidemic hiccup. Evidence of immunity appeared in 3 of these. The previous injection had consisted of dead bacteria, and the injection to which increased resistance was developed consisted of active filtrates. In no instance was increased resistance to the dead bacteria noted following injection of filtrates.

Susceptibility to dead bacteria was noted in animals that had remained free from symptoms following repeated inoculation of active filtrates. Thus, 1 rabbit that had developed spasms of the diaphragm following the first injection of a filtrate of the heated broth culture remained free from symptoms following 3 subsequent injections of the same filtrate, but developed spasms of the diaphragm following the injection of washed, heat-killed bacteria. The shortest interval between injections in the animals that showed immunity was 4 days, the longest 14 days. Increased susceptibility was observed when the injections were repeated in from 7 to 48 hours. This occurred whether the preceding injection was active or inert, and after the injection of sodium chlorid solution. Moreover, evidence of a changed reactivity of the animal was often noted in these early reinjections. The animals, instead of developing spasms of the diaphragm and tremor of the masseters with slight or moderate hyperpnea, often developed extreme hyperpnea, sometimes resulting

in death, without developing spasms of the diaphragm or tremor of the masseters. Certain rabbits were particularly resistant, remaining free from symptoms following repeated injections of active filtrates, dead bacteria, and live cultures; others developed spasms of the diaphragm following as many as 3 repeated inoculations (rabbit 3507). Four rabbits, 1 with marked spasms of the diaphragm, that had recovered from inoculations of live cultures, were found as susceptible to active filtrates and suspensions of dead bacteria as normal controls.

No well marked evidence of immunity to infection on intracerebral inoculation of live cultures was noted following repeated intracerebral injections of filtrates or dead bacteria. This was true whether the same or a different hiccup strain was injected.

Interpretation of these results was difficult on account of the variation in susceptibility of rabbits and the instability of the substance that incites spasms of the diaphragm and tremor of the masseters in the filtrates, dead bacteria and in cultures. Several points, however, appear to be demonstrated by these experiments.

The antigenic power of the filtrate of the dead bacteria and live cultures of the hiccup strains, on intracerebral inoculation, was of a low order, protection being afforded only against filtrates in which the quantity of poison was small. Loss of the property to produce the specific poison in filtrates and dead bacteria was accompanied by loss of immunologic specificity. Immunity appeared only when the antigen and test substance were identical, or at least of the same strain. Repeated inoculations of active filtrates or dead bacteria of freshly isolated strains failed to protect against the toxic substance in the filtrate of the old meat-infusion cultures and against infection by the corresponding live streptococcus, which had lost the power of producing characteristic symptoms.

CONCLUSIONS

The streptococcus of epidemic hiccup has been found to produce a substance or complex of substances which on inoculation into animals produces spasms of the diaphragm sometimes associated with tremor and twitchings of the masseters and other muscles. It is demonstrable in filtrates, in the clear centrifugalized broth, and in the washed dead bacteria of young cultures at the time the living streptococcus produces like symptoms in animals, and disappears from these as the living bacterium loses this power from artificial cultivation. The symptoms and lesions produced were essentially alike, except as to duration and extent, following injections of active filtrates, suspensions of dead bacteria, and the living organism. Hence, the specific localizing power with the production of this highly characteristic syndrome would seem to be due to a chemical substance produced either by the streptococcus or during the reaction incited in the host.

That persistence of spasms of the diaphragm in epidemic hiccup is dependent on the continued production of this substance either in the lesions in the central nervous system, in the focus of infection in the

throat, or elsewhere, is indicated by the facts that characteristic effects were produced by exceedingly small amounts of the substance in highly diluted filtrates on intracerebral inoculation and by intravenous injections of larger amounts, and that the duration of symptoms was shortest following injection of filtrates, longer following the introduction of the dead bacteria, and longest following inoculation of the living streptococcus.

The substance disappears promptly in actively growing cultures, such as glucose-brain broth, but tends to remain longer in slowly growing cultures, such as meat infusion, especially when under partial oxygen tension. No method has yet been found which will indefinitely maintain the power of the hiccup streptococcus to produce this highly characteristic effect. In filtrates of glucose-brain-broth cultures, it has been found resistant to heat (60 C. for 1 hour and 100 C. for 10 minutes), to neutralization to fuchsin and reacidification. It is not dialyzable, but is promptly removed by absorbing agents, such as animal charcoal. With few exceptions, it has been found to disappear in filtrates kept in the ice-chest, and in the heat-killed bacteria in dense suspension in sodium chlorid solution, but is retained in similar suspensions in formalin-killed organisms and when the living streptococci are preserved in dense suspension in glycerol and sodium chlorid solution and kept in the refrigerator.

Experiments in immunization indicate that the antigenic power of these filtrates, dead bacteria, and living streptococci, is of a low order, a fact in keeping with the low virulency of this streptococcus, and that as specific localizing power is lost, specific immunologic properties disappear.

ATYPICAL TYPHOID FEVER WITH SLOWLY AGGLUTINABLE TYPHOID BACILLUS IN A PERIOSTEAL LESION

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That organisms of the coli-typhoid group may invade subcutaneous tissues and produce suppuration is well known. Experimentally, Benians¹ has shown that in coli-typhoid septicemia of rabbits, the organisms may enter a subcutaneous fixation abscess of sterile gum tragacanth, escaping from the vessels without leaving any gross lesion of the wall. Such facility of migration was not observed in the case of *Staphylococcus aureus* and other bacteria. These experiments suggest that similar conditions may obtain in man, and they were found in the recent case of acute purulent myositis of Terada.² Although the organism in these atypical lesions often shows no deviation from type, a few cases, like our own, have revealed atypical organisms either culturally or serologically, so that unless careful studies are made, the true nature of the organism may be overlooked. The main variation of our organism is that it is slowly agglutinable, although prolonged incubation for 6 hours at 37 C. and over night in the icebox leads to its complete agglutination. This fact again emphasizes the irregularities of these organisms, as pointed out by Pfeiffer and Kolle.³

J. D., aged 34, admitted on account of cough, fever, weakness and pain in the leg, said he had been in good health while working as a section hand in a railway camp until 3 weeks before coming to the hospital. At this time he developed cough and nosebleed. On the second day he became nauseated and vomited frequently. After one week, until admitted, he had been sleeping in box cars or walking the streets in the day time. He had eaten with good appetite, but his funds limited him to one meal a day. Four days before admission he noticed a dull, aching pain in the right leg and thigh, which gradually increased until he was no longer able to get about. He denied having had any disturbance of the bowels either in the form of constipation or diarrhea. He thought he had lost weight and was very weak; he gave no history of having had typhoid or having been vaccinated against typhoid; no military service; he was exposed to lice in the bunk house, but his clothes were not infested and there were no scratch marks.

When examined he showed the appearance characteristic of typhoid: face flushed and dusky, with slight conjunctivitis, dry tongue; sordes on lips and

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¹ Brit. Jour. Exper. Path., 1921, 2, p. 276.

² Jour. Am. Med. Assn., 1917, 64, p. 2101.

³ Ztschr. f. Hyg. u. Infektionskr., 1896, 21, p. 203.

tongue, and extensive herpes facialis; spleen palpable at costal margin; right tibia was tender throughout its length, two areas each about 5 cm. long were more tender than others and in one region there was local heat with no redness; a few fine crackling râles at the base of both lungs; temperature 39.5 C.; pulse 105; respiration 30.

For the next five days the temperature ranged from 40 to below 36 C., going a little lower each day, with a pulse that averaged 90; from the sixth day to the twelfth, the temperature remained above 37 C. and going as high as 39.5, and the pulse averaged above 90; respiration remained at 20 throughout; on the fourteenth day the temperature went below 37, and after the twenty-fourth remained so.

With defervescence the mouth became moist, appetite returned, and the mind became brighter. The blood pressure was low, being systolic 105, diastolic 60 at admission; the urine showed only a trace of albumin on admission, which disappeared on the eleventh day; leukocytes numbered 10,200 on admission; hemoglobin, 80 per cent.; blood cultures (aerobic and anaerobic) taken at the time were negative; agglutination tests with *B. typhosus* and *B. paratyphosus* A and B were negative; there was no plasmodium malariae; on the ninth day in the hospital and again during the fifth week the leukocytes were 8,800; the differential count was normal. On recurrence of fever, blood cultures were repeated with negative results; stool and urine cultures also were negative.

On the fourteenth day in the hospital, a small amount of pus was removed from a tender area on the shin, and typhoid organisms were found in the smears and grown on blood agar. With the recovery of this organism, the diagnosis of typhoid fever, which had been proposed, was established. When subsequent developments showed that we were dealing with an unusual infection and it was thought desirable to recover the organism again, the fluctuating area on the shin had completely disappeared, and it was impossible to obtain a second culture.

On the twenty-second day in the hospital, the roentgenogram showed two regions of erosion on the surface of the tibia, and a small crater could be felt in the surface of the tibia at the point indicated.

Bacteriologic Examination.—Following the isolation of the gram-negative, actively motile bacillus from the tibial abscess, a careful study of its cultural and serologic characteristics was made. It was a short rod of the size and shape of the typhoid bacillus, easily stained, and growing well under semi-anaerobic conditions.

On agar after 24 hours at 37 C., there were abundant, flat, glistening, smooth, translucent, undulate and finely granular colonies; no odor; diffuse cloudiness in broth; on gelatin, the growth was undulate and best at the top, developing, however, along the entire track of the needle; no liquefaction; in litmus milk a slight acidity was produced on the fourth day, and on the fourteenth day the milk was distinctly alkaline, no coagulation; nitrate broth was reduced in 24 hours at 35 C., while no diastatic action was observed on starch-agar plates; no indol, and the Voges-Proskauer test was negative. The reactions of various carbohydrates in phenol-red broth were:

Glucose	Acid, no gas	Maltose	Acid, no gas
Levulose	Acid, no gas	Raffinose	No change
Galactose	Acid, no gas	Dextrin	No change
Arabinose	Trace	Inuline	No change
Rhamnose	No change	Mannite	No change
Xylose	Slightly acid	Glycogen	No change
Lactose	No change	Salicin	No change
Saccharose	No change		

On the basis of its general characteristics, the group number of the organism is 222,233,203.

Serologic Examination.—As stated, the patient's serum failed to agglutinate stock *B. typhosus* and *B. paratyphosus* A or B, while it agglutinated the newly isolated organism in a dilution of 1:8 and slightly in a dilution of 1:16, after several hours at 37 C. At this time 4 different antityphoid serums were prepared for macroscopic agglutination tests. In none of these serums was agglutination observed following 1 to 2 hours at 37 C., but an absorption test proved to be positive. Numerous transfers were made on ordinary plain agar, but the organism failed to be agglutinated after 1 to 2 hours at 37 C. Three rabbits were injected intravenously with live cultures of the bacillus, starting with a dose of 0.1 c.c. Two died during the course of immunization, but the third animal after 4 injections with increasing doses gave a serum which in 1 to 2 hours at 37 C., readily clumped the Rawlings strain of typhoid in a dilution of 1:1,000 and slightly in a dilution of 1:2,000, but slowly agglutinated the new strain. In absorption experiments it was found that the organism took all the agglutinins up so that the serum failed to clump the Rawlings strain. The agglutination test was repeated with a longer period at 37 C. At the third hour there was marked agglutination at 1:10 and 1:25, but less in 1:50; at the end of 4 hours, it was marked in tubes up to 1:500; at the end of six hours, agglutination was distinct in the 1:2,000 dilution. Similarly, prolonged incubation led to agglutination of the organism by the stock serum mentioned. Agglutination at 55 C. did not visibly enhance the reaction, and not much difference was observed when 1.8 and 3.6% salt solutions were used instead of 0.85%. These results are in agreement with the work of Ishii.⁴

From these results it is evident that the organism in question is a typhoid bacillus which is relatively inagglutinable. When sufficient time was allowed at 37 C., agglutination occurred. It also appears that there is no direct relation between agglutinability and agglutino-genic properties. Elser and Huntoon⁵ have shown this for meningococci, and recently Torrey and Buckell⁶ found it to be true for gonococci. The conclusion is that the case was one of typhoid with subsequent periostitis and localization in the tibial focus of a bacillus that agglutinated in specific serum only on prolonged incubation.

⁴ Jour. Bacteriol., 1922, 7, p. 39.

⁵ Jour. Med. Res., 1909, 20, p. 371.

⁶ Jour. Immunol., 1922, 7, p. 305.

THE INFLUENCE OF CARBON DIOXIDE ON THE GROWTH OF BACTERIA

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The history of the discovery of oxygen, and the demonstration of its necessity for the maintenance of life of all higher animals is well known. It was thought that bacteria required free oxygen for their growth, until Pasteur¹ showed that some grew only in the absence of oxygen. He thought that these bacteria, the so-called obligatory anaerobes, obtained their oxygen by the fermentation of carbohydrates. From this work and from the work of others, resulted the well-known classification of bacteria according to their respiratory requirements, namely: obligatory and facultative aerobes, facultative and obligatory anaerobes.

That the respiratory requirements of bacteria are not covered by so simple a classification, and that there are many gradations between the respiratory requirements of an obligatory aerobe and an obligatory anaerobe, has been suggested and is indicated by: the adaptation of an obligatory aerobe to grow anaerobically,² the adaptation of an obligatory anaerobe to grow aerobically,³ the effect of heat on the respiration of certain bacteria,⁴ the work of various authors on the partial tension bacteria,⁵ the growth only at certain levels in liquid medium of some bacteria directly isolated from the host, and also of a few saprophytic bacteria,⁶ the growth of bacteria under different tension of respiratory gases,⁷ the relation of the gaseous environment to the digestion of fresh

Received for publication, Sept. 26, 1922.

¹ Pasteur, cited by Kruse: *Allg. Mikrobiol.*, 1910, p. 96.

² Lafforgue, cited by Kruse: *Ibid.*, p. 104.

³ Ferran: *Centralbl. f. Bakteriol.*, I, 1898, 24, p. 28. Bellfonti: cited by Hiss and Zinsser, 1914, p. 458.

⁴ Rabinovitch: *Ztschr. f. Hyg. u. Infektionskr.*, 1895, 20, p. 159.

⁵ Wherry and Oliver: *Jour. Infect. Dis.*, 1916, 19, p. 288; 1917, 20, p. 28. Wherry and Ervin: *Ibid.*, 1918, 22, p. 194. Smith, Theobald: *Jour. Exper. Med.*, 1918, 28, p. 333. Cohen: *Jour. Am. Med. Assn.*, 1916, 67, p. 1302; *ibid.*, 1918, 70, p. 1999; *Jour. Infect. Dis.*, 1918, 23, p. 337. Chapin: *Ibid.*, p. 342. Rockwell and McKhann: *Ibid.*, 1921, 28, p. 249. Fitch, C. P.: *Ibid.*, 1922, 31, p. 231.

⁶ Rosenow: *Ibid.*, 1914, 14, p. 62; *Jour. Am. Med. Assn.*, 1914, 62, p. 1146. Beyerinck: *Centralbl. f. Bakteriol.*, I, 1893, 14, p. 827.

⁷ Fraenkel: *Ztschr. f. Hyg. u. Infektionskr.*, 1889, 5, p. 323; *Centralbl. f. Bakteriol.*, I, 1888, 3, p. 735. Chudiakow: cited by Kruse, *Allg. Mikrobiol.*, 1910, p. 98. Winogradsky, cited by Kruse: *Ibid.*, p. 100. Pasteur: Joubart and Chamberland, cited by Novy, *Centralbl. f. Bakteriol.*, I, 1893, 14, p. 581. Ruediger: *Jour. Infect. Dis.*, 1919, 24, p. 376. Herrold: *Jour. Am. Med. Assn.*, 1920, 74, p. 1716. Swartz: *Ibid.*, 1918, 71, p. 2050. Rockwell: *Jour. Infect. Dis.*, 1921, 28, p. 352.

steer hide,⁸ the necessity of carbon dioxide for the fixation of nitrogen by bacteria,⁹ and that most aerobic bacteria can grow anaerobically if a fermentable carbohydrate be present.¹⁰

Even with this evidence that some organisms, especially when first isolated from the living host, are often more subtle in their respiratory requirements than is indicated by the aerobe-anaerobic classification, there are few who believe this to be the case, and some even deny it.¹¹ while many of those who obtain favorable results with this method, attribute them to either the buffer action of carbon dioxide,¹² or to the better retention of moisture.¹³

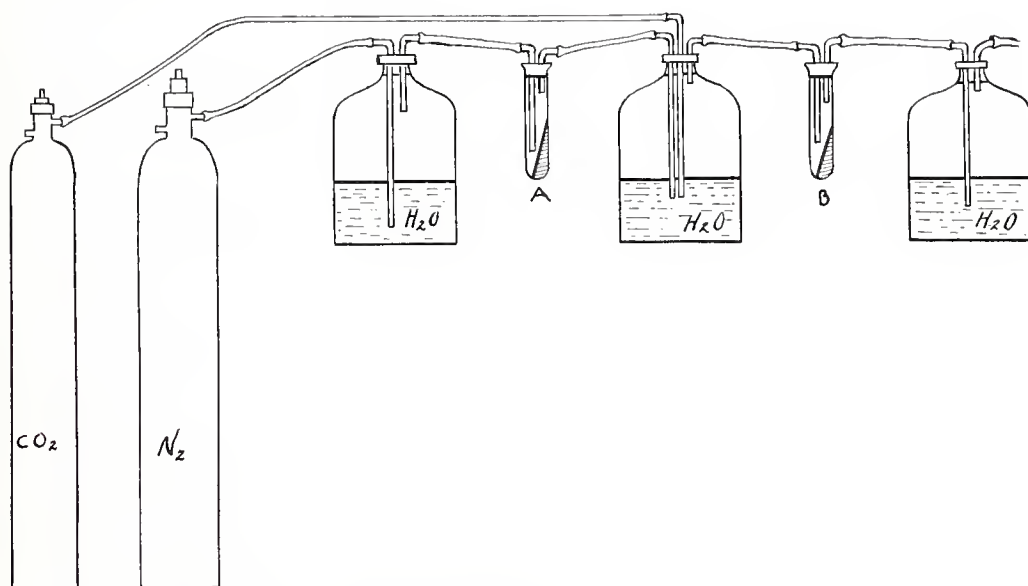


Fig. 1.—Nitrogen was passed through water, then over a culture in tube A. The nitrogen was collected over water, had carbon dioxide added to it, and then passed over a culture in tube B.

In reply, it may be said that the evidence of these critics is not conclusive because in their experiments they have not worked with first generation cultures, or else have failed to control thoroughly the gaseous environment.

This paper is a continuation of work formerly reported,¹⁴ and deals more in detail with the relation of carbon dioxide to the growth of

⁸ McLaughlin and Rockwell: Jour. Am. Leather Chem. Assn., 1922, 17, p. 325.

⁹ Bonazzi: Jour. Bacteriol., 1921, 6, p. 479.

¹⁰ Smith, Theobald: Centralbl. f. Bakteriologie, I, 1895, 18, p. 1.

¹¹ Erickson and Henry: Jour. Infect. Dis., 1922, 30, p. 268.

¹² Kohman: Jour. Bacteriol., 1919, 4, p. 571. Gates: Jour. Exper. Med., 1919, 29, p. 321.

¹³ St. John: Med. Rec., 1919, 95, p. 184. Torrey and Buckell: Jour. Infect. Dis., 1922, 31, p. 125.

¹⁴ Jour. Infect. Dis., 1921, 28, p. 352.

bacteria, freshly cultivated from living tissue and to the growth of bacteria which have become acclimated to artificial mediums.

The following experiments show how certain bacteria grow in an environment (1) completely devoid of oxygen and carbon dioxide (even the small amount of carbon dioxide liberated by the bacteria), and (2) devoid of oxygen but with carbon dioxide present.

METHOD

The following means were used to produce this gaseous environment.

1. A rapid current of moist nitrogen (99½% nitrogen, 0.5% water obtained from an air reduction bomb) was passed over a culture, as shown in figure 1. This not only placed the organism in an atmosphere of nitrogen, but also removed all carbon dioxide as formed. The nitrogen was collected, had carbon dioxide mixed with it, and was then passed over a second culture. The gases were passed through water to wash and moisten them.

2. The air in culture tubes was displaced with nitrogen, and subsequently some of the nitrogen was displaced with 10% sodium hydroxide solution in order to absorb the carbon dioxide as shown in figure 2.

3. A series of inoculated tubes had placed on the cotton stopper 0.5 cc of a 20% solution of pyrogalllic acid, and then added to each tube, respectively, 1, 2, 4, 6, 8, etc., cc of a 10% sodium hydroxide solution. The tubes were then sealed with a soft rubber stopper and incubated upside down.

4. Partial tension environment was produced by growth in tandem with *B. subtilis*.

The medium used was of several types (a) a sugar-free meat extract 2% agar, 0.5% sodium chloride, 0.3% acid to phenolphthalein, and P_H 7.4, (b) a sugar-free meat juice, 2% agar of a P_H 7.2 plus 10% ascites fluid; (c) a sugar-free meat juice, 2% agar of a P_H 7.2 had added to it 5% rabbit defibrinated blood; (d) a meat juice agar of a P_H 7.2 had added to it 1% dextrose and 10% ascites fluid; (e) a meat juice agar plus 5% glycerol, 1% acid to phenolphthalein.

Exper. 1.—The bacterium used in this instance was a member of the *B. subtilis* group, isolated from a steer hide.¹⁵ It was cultivated on the sugar-free agar with the result that it grew aerobically, under a stream of nitrogen plus carbon dioxide; and at partial tension; but it did not grow under a stream of nitrogen, or under pyrogalllic acid and alkali.

Exper. 2.—A saprophytic tubercle bacillus was inoculated on the 1% acid glycerol agar. There was a good growth aerobically and also under the stream of nitrogen plus carbon dioxide; but no growth occurred under the stream of nitrogen, or under pyrogalllic acid and alkali.

Exper. 3.—*B. proteus*, isolated from a steer hide¹⁵ was inoculated on the sugar-free agar and placed under the various conditions. Growth occurred aerobically, under nitrogen plus carbon dioxide, and under pyrogalllic acid and alkali with a small amount of alkali; but no growth occurred if the amount of alkali was increased to 4 cc or more, or when a stream of nitrogen was passed over it.

Exper. 4.—*Staphylococcus aureus*, isolated from a boil, was inoculated on the sugar-free agar, and then placed under the various conditions. A good

¹⁵ McLaughlin and Rockwell: Jour. Am. Leather Chemists Assn., 1922, 17, p. 325.

growth occurred aerobically, only a fair growth under nitrogen plus carbon dioxide, but no growth occurred under a stream of nitrogen or under pyrogallie acid and alkali.

Exper. 5.—An anaerobe, *B. welchii*, isolated from human feces, was inoculated on sugar-free agar and then placed under the various gaseous conditions. A good growth occurred under a stream of nitrogen plus carbon dioxide and

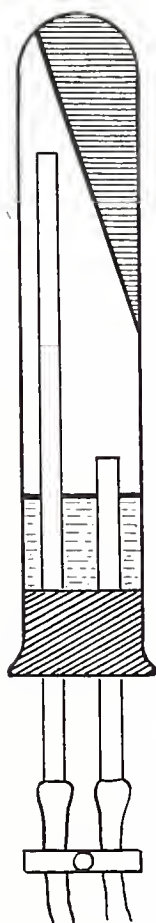


Figure 2

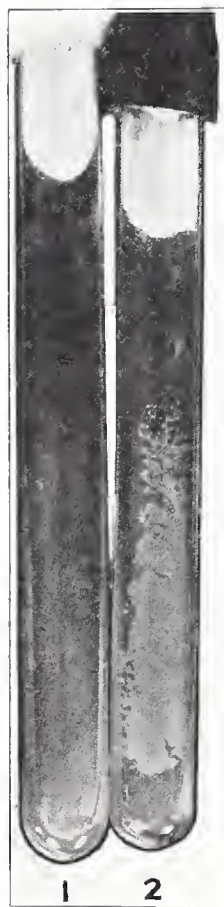


Figure 3

Fig. 2.—The air was displaced with nitrogen, and subsequently some of the nitrogen was displaced with a 10% solution of sodium hydroxide. The tube was incubated upside down.

Fig. 3.—This illustration shows the growth of meningococcus on ascites agar in tube 1, aerobically, and in tube 2, partial tension as produced by *B. subtilis*.

under pyrogallie acid and alkali, with a limited amount of alkali; but an increased amount of alkali to 4 cc or more inhibited the growth; also no growth occurred under a stream of nitrogen, nor under aerobic conditions.

Exper. 6.—An obligatory anaerobe isolated from fresh steer hide was subcultured on sugar-free agar, and then placed under the various gaseous conditions. A good growth occurred under nitrogen plus carbon dioxide, and also

under pyrogallic acid and alkali; but an increasing amount of alkali decreased the amount of growth. There was a slight growth even with 8 cc of alkali present. Under a rapid stream of nitrogen no growth occurred.

Exper. 7.—Pus from a knee joint, when examined in a smear, showed a gram-positive diplococcus, which later proved to be a pneumococcus. Tubes of the sugar-free ascites agar were inoculated with this pus and incubated under the various conditions. Growth occurred under a stream of nitrogen plus carbon dioxide; under nitrogen plus carbon dioxide; under pyrogallic acid and

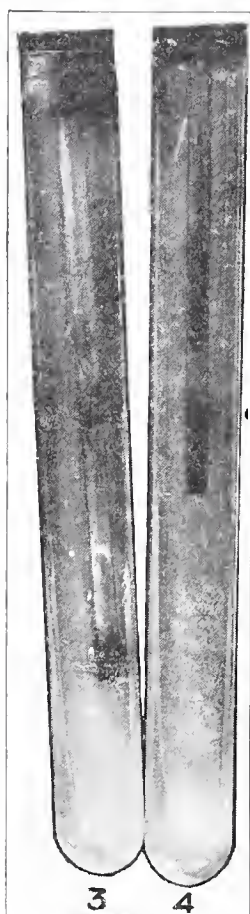


Figure 4

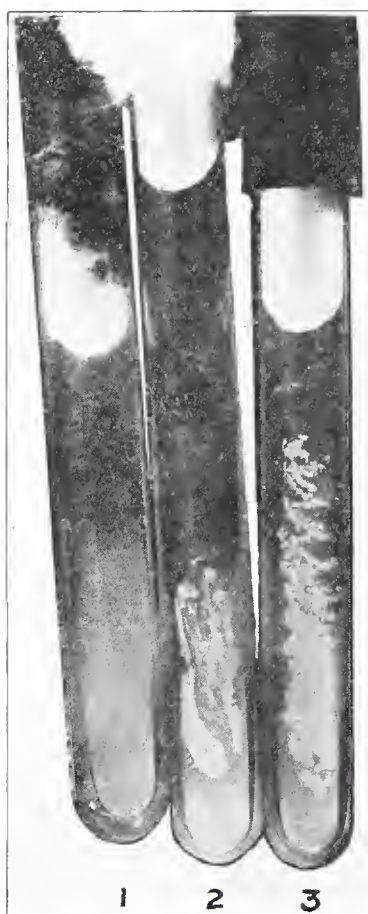


Figure 5

Fig. 4.—This is the same as fig. 3, except that tube 3 was under a stream of nitrogen, and tube 4 under a stream of nitrogen plus carbon dioxide.

Fig. 5.—This shows the growth of meningococcus on dextrose ascites agar—tube 1 anaerobically; tube 2, aerobically; and tube 3, partial tension as produced by *B. subtilis*.

1 cc of alkali, and at partial tension as produced by *B. subtilis*, but no growth occurred aerobically, under a stream of nitrogen, nitrogen plus 10% sodium hydroxide solution, or excess alkali with pyrogallic acid.

Exper. 8.—Pus from an abscess of a hip joint which contained a streptococcus was inoculated on tubes of ascites agar and incubated under the various

conditions. A heavy growth occurred under nitrogen plus carbon dioxide, and a slight growth occurred under pyrogallic acid with only 1 c.c. of alkali added. No growth occurred aerobically, under nitrogen plus alkali, pyrogallic acid with 4 c.c. or more of alkali, or under partial tension as produced by *B. subtilis*.

Exper. 9.—Spinal fluid containing 2,600 pus cells per c.mm. and on smear intracellular gram-negative biscuit shaped diplococci was inoculated on ascites agar, blood agar, and on dextrose ascites agar (fig. 3 and 4). On the ascites agar, good growth occurred under nitrogen plus carbon dioxide, and under partial tension, as produced by *B. subtilis*, but no growth occurred aerobically under nitrogen plus alkali, or under pyrogallic acid plus alkali. On the blood agar, no growth occurred under aerobic, or under pyrogallic acid and alkali; but there was a heavy growth under partial tension. As shown in figure 5, on dextrose ascites agar growth occurred aerobically, and at partial tension, but no growth resulted anaerobically as produced by pyrogallic acid and alkali.

TABLE 1

GROWTH OF VARIOUS ORGANISMS ON SUGAR-FREE MEDIUMS UNDER DIFFERENT GASEOUS ENVIRONMENTS

	Aerobic	Under a Current of Nitrogen	Under a Current of Nitrogen and Carbon Dioxide	0.5 C c of a 20% Pyrogallic Acid Solution, NaOH 10%				Partial Tension
				1 C c	2 C c	4 C c	6 C c	
<i>Bacillus subtilis</i>	+++	—	++	—	—	—	—	++
<i>Tubercle bacillus</i> (saprophyte).....	+++	—	+++	—	—	—	—	
<i>Bacillus proteus</i>	+++	—	++	++	+	—	—	
<i>Staphylococcus aureus</i> ...	+++	—	+	—	—	—	—	
<i>B. welchii</i>	—	—	++	+	±	—	—	
Anaerobe from hide.....	—	—	++	++	+	+	±	
Pus from knee joint containing pneumococcus..	—	—	+++	++	—	—	—	++
Pus from hip abscess containing streptococcus*..	—	—	+++	+	—	—	—	—
Spinal fluid containing meningococcus*.....	—	—	++	—	—	—	—	+++

Amount of growth is indicated by + signs.

* Also — under N₂ + Alk.

This cultivated gram-negative diplococcus was agglutinated by polyvalent meningococcus serum at dilution of 1:200. It also produced acid on glucose and maltose, but did not ferment levulose, galactose, mannose, saccharose, lactose, raffinose, dextrin, inulin, glycerol, erythrite, dulcitol, mannitol, arabinose and isodulcitol. In other words, this was a typical strain of meningococcus.

Table 1 summarizes the results of these experiments.

DISCUSSION

It is apparent that many, if not all, obligatory aerobes are not obligatory, but are able to grow without oxygen, providing the proper tension of carbon dioxide is present.

Anaerobic life is probably more than life without oxygen, carbon dioxide being needed in some if not in all instances.

Bacteria using carbon dioxide for growth may become sensitive to oxygen, and the greater this sensitivity the more they approach to being obligatory anaerobes. It is just before the strict anaerobic group is reached that the partial tension strains exist.

Partial tension strains are of two types. One which needs carbon dioxide for growth, and is indifferent to oxygen, and another which needs carbon dioxide for growth but cannot stand a large amount of oxygen.

SUMMARY

When all oxygen and carbon dioxide was removed (even the carbon dioxide produced by the bacteria) growth was prevented of *B. subtilis*, a saprophytic tubercle bacillus, a staphylococcus, *B. proteus*, *B. welchii*, an anaerobe from a steer hide, a streptococcus, a pneumococcus, and a meningococcus; but when only oxygen was removed and carbon dioxide was added, growth occurred in all instances.

The growth of bacteria under pyrogallic acid and alkali was interfered with when too much alkali was added, probably due to the absorption of all carbon dioxide as formed. This is further substantiated by the parallel experiments under a stream of nitrogen.

A streptococcus when first cultivated from the host required the presence of carbon dioxide and could not stand any appreciable amount of oxygen. But a pneumococcus and meningococcus when first cultivated from the host required carbon dioxide, but could stand a fairly large amount of oxygen.

These facts again prove that many bacteria when first cultivated from the host need carbon dioxide for rapid growth, and that some of these cannot stand a high concentration of oxygen.

A COMPARATIVE STUDY OF PRECIPITINOGEN AND PRECIPITIN CURVES

WITH ESPECIAL REFERENCE TO THE LATER HISTORY OF
THE PRECIPITIN CURVE

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HISTORICAL REVIEW

Soon after Kraus'¹ discovery of the precipitin reaction in 1897, the fact was noted² that precipitinogen, following its introduction into the body, did not disappear from the blood stream until precipitin had reached a relatively high concentration, and that antigen and antibody, therefore, intermingled freely in the circulation without combining. This phenomenon immediately became the subject of considerable painstaking research out of which have come at least three suggested explanations

suggestion that it could be
satisfying the belief that the
partly in a combined state,

ERRATA

Vol. 30, page 555, in legend for Figure 3, 0.7 should
be 0.1; and on page 556, in legend for Figure 5, 0.1
should be 0.7.

is to this explanation; first,
are governed by the law of
not; and, second, that the
should show a detectable
Lay and Rusk,⁵ have shown
the close similarity between
believing that other serum

concerns exert a protective action, inhibiting precipitation.

Von Dungern⁶ made a thorough investigation of the same problem, coming to the conclusion that a multiplicity of antigens was responsible for the antibody response, which latter, of course, was also polyvalent. This is undoubtedly the case when a serum is utilized as an antigen, since it contains a number of proteins each having antigenic properties. According to his interpretation, therefore, while precipitinogen and precipitin actually may exist side by side without interaction, homologous precipitinogen and precipitin may not.

Obviously the next logical step was the use of a single purified protein as an antigen. Weil⁷ employed crystallized albumin from the hen's egg as antigen

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¹ Wien. klin. Wchnschr., 1897, 10, p. 736.

² Ascoli, M.: München. med. Wchnschr., 1902, 49, p. 1409; Fornet and Muller: Ztschr. f. biol. Technik u. Methodik, 1908, 1, p. 201; Hamburger and Moro: Wien. klin. Wchnschr., 1903, 16, p. 445; Linossier and Lemoine: Compt. rend. Soc. biol., 1902, 54, p. 85; Michaelis and Oppenheimer: Arch. f. Anat. u. Physiol., 1902, quoted by Nuttall, Blood Immunity and Relationship, 1904, p. 130; Physiol. Abt., Suppl., 336; Obermayer and Pick: Wien. klin. Rundschau, 1902; Rostoski, L.: München. med. Wchnschr., 1902, 49, p. 70.

³ Centralbl. f. Bakteriöl., 1903, 34, p. 259.

⁴ Jour. Exper. Med., 1913, 17, p. 396.

⁵ Quoted from Zinsser, Infection and Resistance, 1918, p. 267.

⁶ Centralbl. f. Bakteriöl., 1903, 34, p. 355.

⁷ Jour. Immunol., 1916, 1, p. 1.

and was unable therewith to detect the coexistence of antigen and antibody. This failed of confirmation, however, at the hand of Bayne-Jones,⁸ who, using crystallized egg albumin and edestin, carefully repeated Weil's work and demonstrated the coexistence of precipitinogen and precipitin with these pure substances. He also furnished additional support for the belief of Zinsser and Young⁴ in the protective action of certain serum colloids by showing that the presence of egg albumin in proper proportion could prevent the precipitation of a human serum by an antihuman serum.

The latter view probably holds most general favor at the present day.

Hektoen⁹ has made a study of the precipitin curve in rabbits. For this purpose he employed as antigens human and chicken serum and chicken and sheep whole blood in doses of 5 cc, 10 cc, and 15 cc on successive days. He found precipitin appearing after from 2 to 5 days, usually about the second or third, and reaching its crest in from 9 to 14 days following the final administration of antigen, the twelfth day being approximately the average.

In subsequent studies, the same author traces the course of the precipitin curve in rabbits treated before, during, and after the immunizing period with subcutaneous injections of benzene and toluene, substances destructive of the blood-forming tissues, and also in normal and allergic¹⁰ rabbits. His findings here with regard to the normal curves corroborate his previous work, but precipitin production began on the average a day or two later. While he made no deliberate investigation of antigen persistence, he discovered in some rabbits injected with 30 cc of sheep blood that the antigen was demonstrable after from 10 to 12 days, and in one case as long as 20 days after injection.

Longcope and Rackemann,¹¹ Longcope and Mackenzie,¹² and Mackenzie and Leake¹³ studied the relation of precipitin formation and antigen elimination to serum disease susceptibility, and in so doing have furnished additional data on the relations of the antigen and antibody curves. These investigations were carried out on patients injected with horse serum (antibacterial and antitoxic). Longcope and Mackenzie found it possible to separate human beings into two groups: (1) those who experienced severe serum sickness, produced precipitin in high concentration, and in whom precipitinogen disappeared with relative rapidity, and (2) those who showed no symptoms of serum disease, produced little or no antibody, and retained the injected horse serum almost indefinitely.

Mackenzie and Leake¹³ have recently elaborated the study of these two groups and with regard to the antigen and antibody curves record in more detail the results of Longcope and Rackemann and of Longcope and Mackenzie. They ascertained that in group 1 precipitinogen was demonstrable for from 18 to 40 days after final injection, while precipitin began to appear after from 8 to 15 days and reached its crest after from 9 to 40 days. Two of their precipitin curves which are carried to the end point (the point of final disappearance of the antibody) terminate after 67 and 58 days, respectively.

Longcope and Mackenzie¹² in a single paragraph record their observation that, following the intravenous injection of 5 cc of horse serum per kilo body weight into rabbits, presence of the injected antigen could be detected for from

⁸ Jour. Exper. Med., 1917, 25, p. 837.

⁹ Jour. Infect. Dis., 1914, 14, p. 403; 1916, 19, pp. 69 and 737; 1917, 21, p. 279.

¹⁰ Rabbits which had been immunized against one antigen (sheep blood), and which, subsequent to the disappearance of homologous antibodies, were immunized against a different antigen (horse blood), developed thereupon antibodies against both sheep and horse blood.

¹¹ Jour. Exper. Med., 1918, 27, p. 341.

¹² Proc. Soc. Exper. Biol. & Med., 1920, 17, p. 133.

¹³ Jour. Exper. Med., 1921, 33, p. 471.

7 days to 3 weeks, while precipitin appeared after from 6 to 10 days and persisted for from 4 to 7 weeks.

Alexander¹⁴ in a recent comparative study of precipitin response following, respectively, intraspinal and intravenous injections of similar amounts of horse serum, found that precipitin production began only after from 7 to 9 days. No observations were made on the persistence of antigen. This investigator obtained remarkably high titers considering the small amounts of antigen injected. Administering 0.5 cc of horse serum intraspinally to one rabbit and simultaneously an equal amount intravenously to another, he found that while antibody production usually began about the same time by both methods, the former produced uniformly higher titers, which typically ran as high as 1:10,000, the latter producing titers of from 1:500 to 1:5,000.

PURPOSE OF THIS INVESTIGATION

Excepting the work of Longcope and Rackemann, of Longcope and Mackenzie, and of Mackenzie and Leake, already cited, no efforts have been made to correlate the persistence of precipitinogen with the time and rate of appearance of its homologous antibody. It was the original purpose of this investigation to make a study only of the persistence of antigen, but since the same experiments could be used for the study both of the antigen and antibody curves, the scope of the work was enlarged to make a comparative study of the two.

TECHNIC

Materials.—Sheep serum was used as an antigen and rabbits were used as test animals. A single healthy sheep furnished all the serum used for antigenic purposes. Fourteen rabbits were studied, all young healthy animals, varying in age approximately from 10 months to 2 years, and in weight from 1,900 gm. to 3,500 gm., all except 2 weighing between 2,200 and 2,900 gm. Rabbit 3 had received 4 or 5 injections of thyroid extract intravenously several months previously, and somewhat earlier had been immunized against *B. typhosus*. Rabbit 17 lost her identification tag and had probably been immunized to *B. typhosus* previously. None of the other animals had ever been employed for experimental purposes.

Treatment of Animals.—The treatment of the test animals is for convenience given in condensed form in table 1.

Rabbits 3, 6, 10, 11, 12 and 13 were each given 5 cc of sheep serum intravenously regardless of body weight. In these the antigenic curves were determined, but not the antibody curves. Five more, rabbits 15, 17, 18, 19 and 20 received 5 cc, 7.5 cc and 10 cc on the first, third and fifth days, respectively, the first dose being intravenous, the second and third doses intravenous or intraperitoneal, varying in different experiments. The last three, rabbits 21, 22 and 23 were given 5 cc intravenously and 10 cc and 15 cc intraperitoneally on the first, third, and fifth days, respectively. All the animals were bled periodically,¹⁵ as shown in the accompanying curves, and their serums were titrated (1) against antish sheep serum for determination of their precipitinogen content, and (2) with normal

¹⁴ Jour. Exper. Med., 1921, 33, p. 471.

¹⁵ All bleedings date from the final injection of antigen.

sheep serums to determine the precipitin content. From the results of these periodical titrations the curves were constructed. Never more than 5 cc, and usually only 2 or 3 cc, were drawn at a single bleeding.

Antiserums for Detection of Antigen.—For production of the antiserums used in the detection of the antigen in the circulation of the animals, fowls were at first utilized. Six 7 months old Rhode Island red roosters were immunized, 3 by weekly doses of 4 cc, 7 cc and 10 cc of sheep serum, 3 by successive daily doses of 5 cc, 7 cc and 10 cc, the first 2 being intravenously administered, the last intraperitoneally. Little difference could be observed in the resulting titers by the two methods. The titer of every antiserum obtained by both methods was 1:20,000, except in 1 case in which an animal, having been immunized by weekly doses, was immunized a second time by 3 daily doses of like amounts. The titer following the first immunization was 1:20,000, the second 1:50,000. As recommended by Hektoen,¹⁰ the fowls were bled from 9 to 12 days after the last injection.

TABLE 1
TREATMENT OF TEST ANIMALS

No. of Rabbit	Weight of Rabbit in Grams	Titer of Antiserum Used for Detection	Injections of Sheep Serum		
			1st Day	3d-5th Day	5th Day
3 (♀)	2,442	1:20,000	5 cc iv*
6 (♀)	2,703	1:50,000	5 cc iv
10 (♂)	2,915	1:16,000	5 cc iv
11 (♂)	2,547	1:16,000	5 cc iv
12 (♂)	2,673	1:16,000	5 cc iv
13 (♂)	2,900	1:16,000	5 cc iv
15 (♀)	2,951	1:32,000	5 cc iv	7.5 cc iv	10 cc ip
17 (♀)	2,855	1:10,240	5 cc iv	7.5 cc iv	10 cc iv
18 (♀)	1,887	(1: 5,120) (1:32,000)	5 cc iv	7.5 cc iv	10 cc iv
19 (♀)	2,395	1: 8,000	5 cc iv	7.5 cc ip	10 cc ip
20 (♀)	2,642	1: 8,000	5 cc iv	7.5 cc ip	10 cc ip
21 (♀)	2,183	1:10,240	5 cc iv	10 cc ip	15 cc ip
22 (♀)	2,448	1:10,240	5 cc iv	10 cc ip	15 cc ip
23 (♀)	2,818	1: 5,120	5 cc iv	10 cc ip	15 cc ip

* Iv signifies intravenous; ip signifies intraperitoneal.

Chickens were bled usually from the wing veins to the amount of 20 or 25 cc. Fowls have been found by the writer, next to rabbits, the easiest of all laboratory animals to bleed, and they possess the additional qualifications of being reliable antibody producers and having great resistance against ordinary infections.

Fowl-produced antiserums were used in only the first of the 14 experiments here recorded, however. In all of the later experiments rabbits were employed for this purpose for two reasons: 1. Normal precipitins in chicken serum sometimes cause precipitation of rabbit serum when the concentration of the latter is 1:10 or higher. This difficulty is obviated when rabbit-produced antiserums are used for detection of the sheep serum in the test animal's serum, no evidence of iso-precipitins having been encountered, and the persistence of antigen can be determined up to its point of disappearance. 2. Bayne-Jones,⁸ as has already been stated, found that the presence of another colloid (egg albumin) might inhibit the precipitation of an antigen by its specific antibody. Chicken-produced antiserums would introduce another colloidal system, which might play some rôle in the actual titration.

¹⁰ Jour. Infect. Dis., 1918, 22, p. 561.

Various amounts of sheep serum and various intervals between doses were employed in the production of the antiserums in rabbits, and titers ranging from 1:5,000 to 1:50,000 were obtained. The most satisfactory results were obtained with injections of 5 cc intravenously, and 7.5 cc and 10 cc intraperitoneally, on the first, third and fifth days, respectively, bleeding about the eighth day after the last injection. Larger amounts of administered antigen were not found to produce higher titers, and in some cases produced considerable loss of weight and vitality, with probable delay in maximum antibody output. Smaller doses may sometimes elicit equally higher titers, but the dosage cited has been found uniformly good, except in some cases in which no antibody formation occurred. Such cases as the latter are known to all who have employed rabbits for antibody production.

It should be noted that the study of the persistence of precipitinogen in such animals as these, in which no antibody production followed injection, ought to prove interesting in the light of the work of Mackenzie and Leake,¹³ already cited. Lack of time prevented its inclusion in the present work.

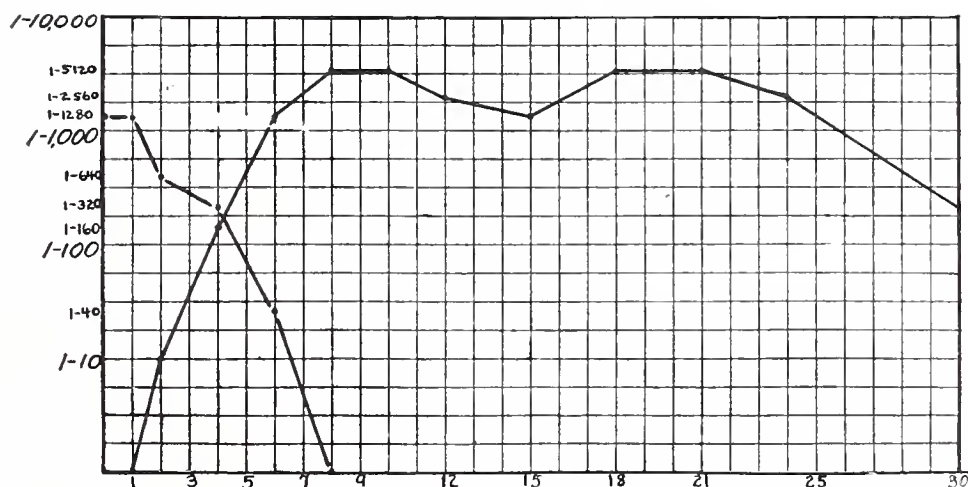


Chart 1.—Rabbit 15. 5, 7.5 cc of sheep serum intravenously, 10 cc intraperitoneally. In charts 1, 2, 3 and 4 the dot and dash line represents the precipitinogen curve, the continuous line the precipitin curve.

The antiserums, produced as outlined, were obtained by bleeding from the median ear arteries (auriculars) of the rabbits.

Technic of Titrations.—In all titrations 0.5 cc of each dilution of the antigen was placed in successive tubes, 0.1 cc of the undiluted antiserum introduced at the bottom of each tube, and the ring test made before the contents were shaken. The tubes were allowed to stand 20 minutes for the ring test and were then thoroughly shaken and allowed to stand for 2 hours for the flocculation test. In the later experiments, readings of the precipitation were made after 6 hours and 18 hours, for reasons which will be given. All titrations were made at room temperature.

The 1:2 dilution was taken as the zero dilution, since, in higher antigenic concentration than this, antigen and antiserum are of equal specific gravity and intermixture results, making the ring test uncertain.

Dilutions of the antigen were made in nearly every case by the Stern-Körte method of successive dilution, this being considered least subject to error. In

the last few experiments, in which a wide range of dilutions was used (16 tubes, ranging from 1:2 to 1:81,920), those from 1:5 to 1:20, inclusive, were made from a 1:5 dilution, those from 1:40 to 1:640, inclusive, from a 1:40 dilution, and those from 1:1280 to 1:81,920, inclusive, from a 1:1,280 dilution. If the entire series had been made from the 1:5 dilution, any slight error in the first few tubes would have become considerable in the higher dilutions.

Controls were set up with every titration, consisting of: (1) 0.5 cc of 0.85% NaCl with 0.1 cc of the undiluted antigen, and (2) 0.5 cc of 0.85% NaCl with 0.1 cc of the undiluted antiserum.

The use of a hand lens (inverted ocular of a microscope is equally satisfactory) was found to make the determination of the limit of flocculation much more certain and was used in all titrations. The tubes were held against a dark background with artificial light falling on them, but not on the background.

EXPERIMENTAL DATA AND DISCUSSION OF RESULTS

The results of the present study are shown substantially in the accompanying graphs.

Explanation of Curves.—Ordinates represent titers, abscissae the numbers of days following final injection of antigen. Decimal curves have been used exclusively. This was found necessary in order to do justice to low titers, without which the end points of curves could not be satisfactorily shown.

It will be observed that in charts 5, 6 and 7, inclusive, there are 3 pairs of curves, each pair consisting of a heavy line and a light line of the same type. These represent, respectively, the highest and lowest dilutions in which precipitation (either ring precipitation or flocculation, as the case may be) occurred. When, after a certain number of days, such precipitation no longer occurred, the particular pair of lines was brought to the zero line (abscissa). The significance of these curves will become apparent in the section on the later history of the antibody curve.

The necessity of using 2 antisera of different titers for detection of the antigen is responsible for the broken curve in chart 3.

DISCUSSION OF RESULTS

It is obvious that the courses of the antigenic curves, and also their respective points of high titer, vary with several factors, chief of which are the variability in body weight (and consequently in blood volume) and the considerable range in titer of the several antisera used for detection of the antigen. It must be remembered that the injected antigen undergoes dilution to the extent of the animal's blood volume. Antigenic titers, therefore, as shown in the charts, represent not actual dilutions of the sheep serum, but dilutions of the sheep-containing rabbit serum. The dilution thus undergone in the rabbit's circulation, as computed from the results of the work of Meek and Gasser¹⁷ on blood volume in rabbits, cats, and dogs, is approximately

¹⁷ Am. Jour. Physiol., 1918, 47, p. 302. These investigators determined the blood volume of rabbits to be an average of 5.44% of the body weight.

1:20. The peaks and end points of the antigenic curves, therefore, are for purposes of comparison with each other of only approximate value, unless corrections are made for these facts and ideal curves constructed from the resulting calculations. This was, in fact, undertaken, but was given up as not feasible, because too many variables in the calculations made the results of little value.

Antigen curves are of value, however, for comparison with their homologous antibody curves.

In every case the antigen curve persists several days after the appearance of precipitin, and in most instances until the latter is present in fairly high titer. Precipitin production has in each instance begun at least by the third day, and in 2 cases within 24 hours. The peak

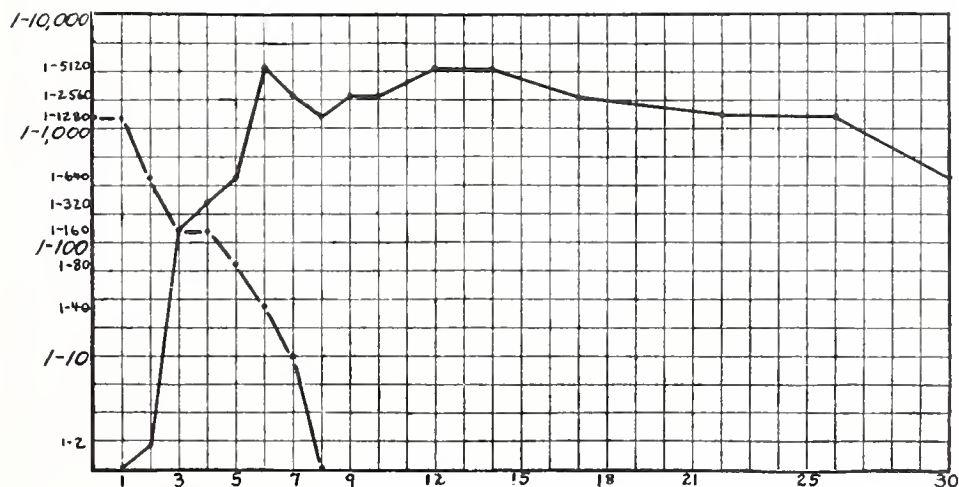


Chart 2.—Rabbit 17. 5, 7.5, 10 c c of sheep serum intravenously.

of the antibody curve occurred in an average time of 10.3 days after the last injection.

The fact is noteworthy that antigenic doses totaling as much as 20 or 30 c c required no greater time for their complete disappearance from the circulation than did the lesser dosage of 5 c c. In fact, the average time in the former cases was less (7.25 days as against 9.66 days). While the data presented are not sufficient to make generalization secure, the writer suggests the probability (which seems to have been borne out in previous work, the records of which do not afford sufficient data on this point) that, in these particular experiments, the larger quantity of injected antigen called forth a more vigorous reaction against the foreign protein; and that, in general, the more severe or

more toxic the dosage, provided it is not sufficient to prostrate or notably weaken the animal, the more rapid will be the elimination of the foreign substance, and the more rapid and powerful the antibody formation.

While there appears to be no exact ratio between the fall of antigen content and the rise of antibody titer, it is evident that the most rapid disappearance of antigen is simultaneous with, or immediately consequent on, the period of abrupt rise in antibody titer.

The double-peaked antibody curves shown in figures 1, 2 and 7 are of interest. Had bleedings been made less frequently, the interval between the last injection and the crest of the curve might have been reported differently. No explanation is offered for this second rise in titer.

It should be noted that the frequent bleedings which were desirable for determining the course of antigen elimination in some cases probably delayed the regular progress of this process and that of antibody formation. Whenever too frequent bleedings seemed to interfere with the well-being of the animal, as evidenced by its lethargy, refusal of food, and general indisposition, they were discontinued for a day or two. It seems likely that in a normal animal which is in a state of perfect health (if such an animal were obtainable), undisturbed by periodic bleedings or otherwise, disappearance of antigen and rise of antibody titer would be represented by smooth curves, proceeding rapidly once they had begun.

As will be seen, the observations of the present study find themselves more in accord with the early results of Hektoen⁹ than with those of Longcope and Rackemann,¹¹ Longcope and Mackenzie,¹² Mackenzie and Leake,¹³ and Alexander.¹⁴ It will be recalled that each of the latter found antigen persisting considerably longer and precipitin much slower in putting in its appearance. In each of these investigations horse serum was employed as an antigen. It is possible that this fact may account for the slower response. Or perhaps it is explainable on the ground that human beings, in general, are less prompt in antibody production than are experimental animals. The latter case, however, does not explain the slower precipitin formation in rabbits obtained by Longcope and Mackenzie and by Alexander.

Time relations between antigen and antibody curves, as described by Mackenzie and Leake, are similar to those indicated in this paper, marked decrease in precipitinogen occurring synchronously with marked increase in precipitin.

Precipitin appeared more promptly after the final administration of antigen in this investigation than in some of Hektoen's⁹ work. This was to be expected, however, since he employed daily antigenic doses, while I injected antigen every other day.

Later History of the Antibody Curve.—As stated in the introduction, the antibody curve does not pursue a consistent downward course from its peak to its point of extinction, but after about 30 to 50 days assumes a decidedly different behavior. If the periods before and after the beginning of this altered behavior may be designated as "primary" and "secondary," respectively, much repetition of words will be obviated.

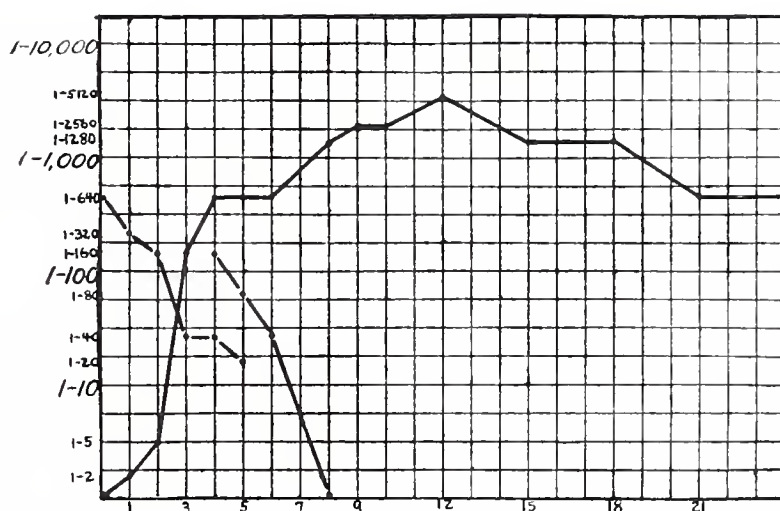


Chart 3.—Rabbit 18. 5, 7.5, 10 c c of sheep serum intravenously.

Before entering on a description of the secondary period, attention should be called to one fact. In the typical forensic precipitin titration, the ring test is read after 15 or 20 minutes at room temperature, the tubes with their contents are then thoroughly shaken, and after 1 hour in a thermostat, or 2 hours at room temperature, the flocculation test is read. In addition, the tubes are sometimes left in the icebox overnight, and the final reading is then made. If these time relations are observed, the ring test and the flocculation test are nearly always in agreement. If, however, the flocculation test is read after, e. g., 6 hours at room temperature, precipitation has occurred in higher dilutions, and after 18 hours at room temperature yet higher. It is approximately complete at the end of this time.

During the secondary period, therefore, readings were made: (1) of the ring test; (2) of the flocculation test after 2 hours, and (3) after 18 hours, and different types of lines, as described in the explanation of the curves, were used for each of these 3 readings. As previously noted, the heavier of each pair of lines (e. g., in the 18-hour curve) represents the highest dilutions in which precipitation occurred, the lighter line the lowest dilutions in which precipitation occurred. Six-hour readings also were made, but, since these would only have been intermediate to the 2-hour and 18-hour, they were omitted in order not to complicate the figures further.

In tables 2 and 3 are shown records for the twelfth-day and fortieth-day titrations, respectively, of rabbit 22, which illustrate the results of typical primary and secondary titrations.

TABLE 2
RECORD OF 12TH-DAY TITRATION OF RABBIT 22

Normal Sheep Serum	Serum of Rabbit 22	0.85% NaCl	Precipitation			
			Ring	2 Hours	6 Hours	18 Hours
0.5 c c 1:2.....	0.1 c c undiluted	++	+	+	+
0.5 c c 1:5.....	0.1 c c undiluted	++	+	+	+
0.5 c c 1:10.....	0.1 c c undiluted	+++	+	+	+
0.5 c c 1:20.....	0.1 c c undiluted	+++	+	+	+
0.5 c c 1:40.....	0.1 c c undiluted	+++	+	+	+
0.5 c c 1:80.....	0.1 c c undiluted	+++	+	+	+
0.5 c c 1:160.....	0.1 c c undiluted	+++	+	+	+
0.5 c c 1:320.....	0.1 c c undiluted	++	+	+	+
0.5 c c 1:640.....	0.1 c c undiluted	++	+	+	+
0.5 c c 1:1,280.....	0.1 c c undiluted	++	+	+	+
0.5 c c 1:2,560.....	0.1 c c undiluted	+	+	+	+
0.5 c c 1:5,120.....	0.1 c c undiluted	+	+	+	+
0.5 c c 1:10,240.....	0.1 c c undiluted	—	—	+	+
0.5 c c 1:20,480.....	0.1 c c undiluted	—	—	—	+
0.5 c c 1:40,960.....	0.1 c c undiluted	—	—	—	—
0.5 c c 1:81,920.....	0.1 c c undiluted	—	—	—	—
0.1 c c undiluted.....	0.5 c c	—	—	—	—
	0.1 c c undiluted	0.5 c c	—	—	—	—

BEHAVIOR CHARACTERISTIC OF THE SECONDARY PERIOD AND COM-
PARISON WITH TYPICAL TITRATION DURING PRIMARY PERIOD

(1) In the last typical titration of the primary period there is precipitation in every tube up to a certain dilution (e. g., from 1:2 to 1:2,560). The quantity of the precipitum increases with the concentration of the antigen, with the exception of the decrease characterizing the so-called proprecipitoid zone which typically includes dilutions between 1:2 and 1:20. Even in these concentrations, however, precipitation is considerable.

(2) An altered behavior is first evidenced in the 2-hour flocculation test. If the 2-hour curves in chart 6, for example, are followed, it is

observed that on the fiftieth day precipitation has occurred in as high a dilution as 1:640, but in no lower a dilution than 1:80. On the sixtieth day, and thereafter, flocculation has ceased altogether during the prescribed 2-hour period, and the 2 curves (representing the upper and lower limits of precipitation) are brought to the zero line (abscissa).

The tubes were allowed to stand 18 hours at room temperature, however, and during this period precipitation occurred within wider limits.

(3) Observed after 18 hours on the fiftieth day (chart 6), precipitation has occurred in every tube between the dilutions 1:40 and 1:5,120, but in no lower dilutions. The 18-hour curves persist much longer than do the 2-hour curves, in this case until the one hundredth day. During this time the lower and higher curves of each pair con-

TABLE 3
RECORD OF FORTIETH-DAY TITRATION OF RABBIT 22

Normal Sheep Serum	Serum of Rabbit 22	0.85% NaCl	Precipitation			
			Ring	2 Hours	6 Hours	18 Hours
0.5 c c 1:2.....	0.1 c c undiluted	+	—	—	—
0.5 c c 1:5.....	0.1 c c undiluted	++	—	—	—
0.5 c c 1:10.....	0.1 c c undiluted	++	—	—	—
0.5 c c 1:20.....	0.1 c c undiluted	++	—	—	—
0.5 c c 1:40.....	0.1 c c undiluted	++	—	—	—
0.5 c c 1:80.....	0.1 c c undiluted	++	—	—	+
0.5 c c 1:160.....	0.1 c c undiluted	++	—	+	+
0.5 c c 1:320.....	0.1 c c undiluted	+	—	+	+
0.5 c c 1:640.....	0.1 c c undiluted	—	—	+	+
0.5 c c 1:1,280.....	0.1 c c undiluted	—	—	—	+
0.5 c c 1:2,560.....	0.1 c c undiluted	—	—	—	+
0.5 c c 1:5,120.....	0.1 c c undiluted	—	—	—	—
0.5 c c 1:10,240.....	0.1 c c undiluted	—	—	—	—
0.5 c c 1:20,480.....	0.1 c c undiluted	—	—	—	—
0.5 c c 1:40,960.....	0.1 c c undiluted	—	—	—	—
0.5 c c 1:81,920.....	0.1 c c undiluted	—	—	—	—
0.1 c c undiluted.....	0.1 c c undiluted	0.5 c c	—	—	—	—
	0.1 c c undiluted	0.5 c c	—	—	—	—

verge, the lowest dilution in which precipitation occurs rising, and the highest dilution in which precipitation occurs falling, until finally precipitation ceases entirely, and the curves are brought to the zero line.

(4) The ring test continues to be "typical" for some time after the beginning of the secondary period (and after the 2-hour curve has ended), except that the quantity of the precipitation is little, if any, greater in the lower dilutions than in the higher. This, of course, is not the case in primary titrations. The upper curve for the ring test gradually descends, but not in conformity with the 2-hour flocculation curve, nor with the 18-hour curve. Within 10 or 20 days after the beginning of the secondary period, however, the ring precipitates are

lacking in the lower dilutions, and thereafter the 2 ring test curves gradually converge.

(5) It is obvious from charts 5, 6 and 7 that the ring precipitate curves follow quite different courses from the flocculation curves and far outlive the latter. At the time of writing none of the ring precipitate curves studied have ended, although all of the flocculation curves are complete. Considerable fluctuation, both of the lower and upper limits of precipitation, will be seen to characterize the curves of the secondary period, especially that of the ring test. No return of the property of flocculation has occurred, however, after it has once disappeared.

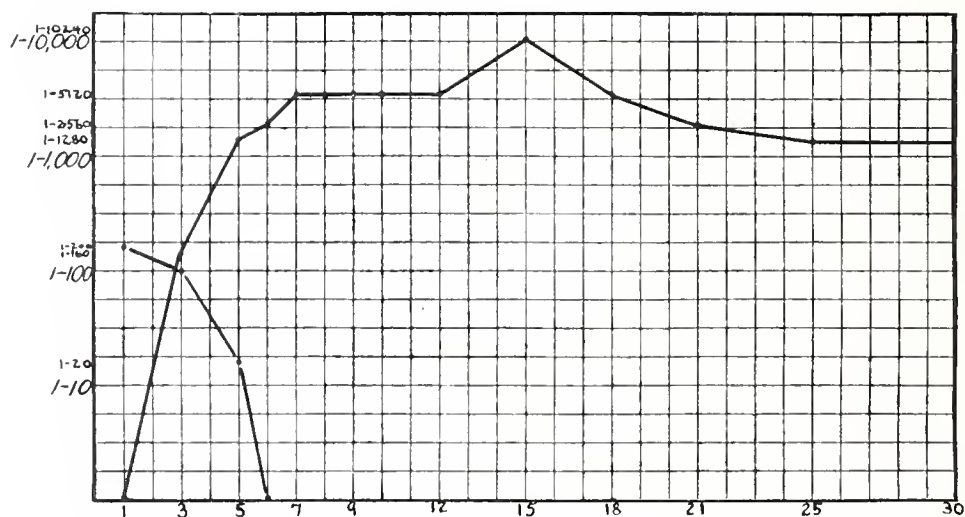


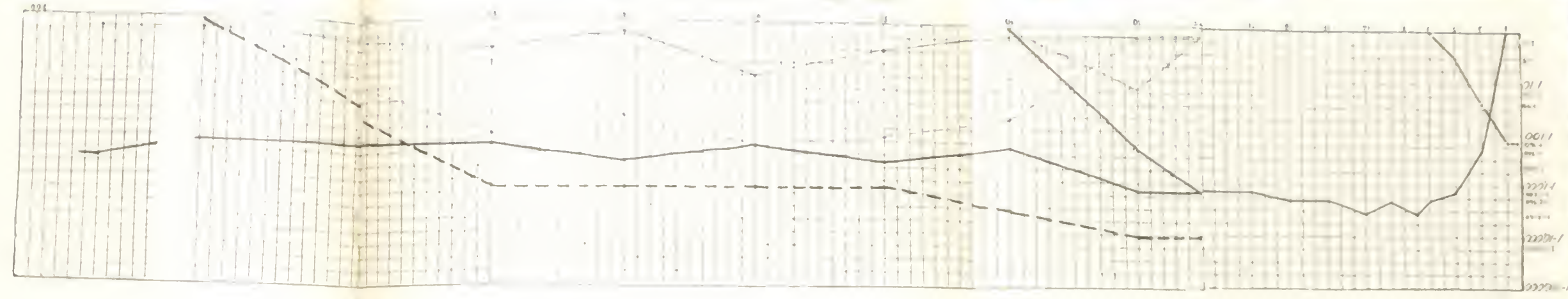
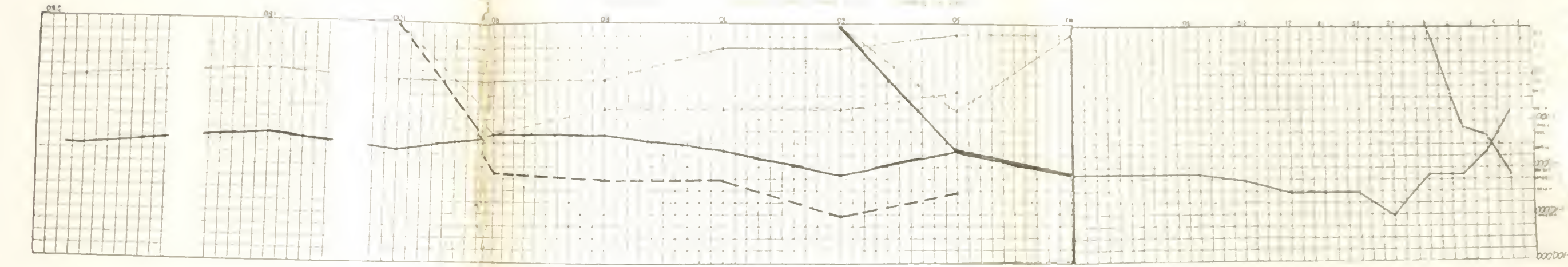
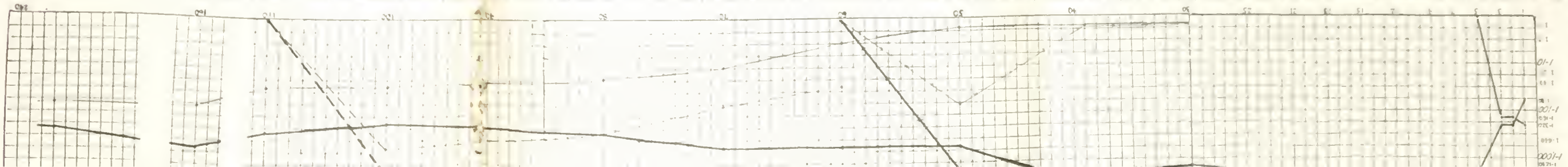
Chart 4.—Rabbit 19. 5 cc of sheep serum intravenously, 7.5, 10 cc intraperitoneally.

The duration of the primary period seems to be relatively independent of the amount of antigen injected and of the body weight.

The same phenomena, as here described for Rabbits 20, 21 and 22, were also observed in rabbits 15 to 19, inclusive, but sufficient data for reporting them were not preserved. Rabbit 23 was being studied simultaneously with rabbit 22, but died on the forty-ninth day.

It is almost needless to say that the control tubes are invariably clear after 18 hours at room temperature.

Conclusions.—The divergence of the ring precipitate curves, and the flocculation curves point to the probability that they represent different processes. This does not, of course, alter the value of the ring test in the forensic precipitin titration, since it has been standardized



to agree with the 2-hour flocculation test, and during the primary period is entirely trustworthy.

No study has been made of the cause or causes underlying the changed character of the secondary titration. One explanation which suggests itself, however, is that a change in the relative proportions of colloidal substances in the antiserum has occurred which has resulted in an alteration of the protective activity of one or more of them.

The long persistence of the positive ring test may also help to fortify the antigen-antibody conception of anaphylaxis. The quantitative relationships between the precipitating power of an antiserum and its ability to sensitize for passive anaphylaxis are familiar to all through the work of Doerr and Russ¹⁸ and others. The chief objection that has been raised to this, however, is that an actively sensitized animal remains sensitive, at least in some degree, much longer than precipitins are demonstrable in its circulation. It is evident, however, from figures 5, 6 and 7 that precipitins continue in slight concentration for a considerable period after final injection; and even after a serum has lost its flocculating power, the positive ring test indicates that it is not yet to be considered a normal serum.

SUMMARY

With the use of sheep serum as an antigen, the precipitinogen-disappearance curve, the precipitin curve, and the interrelations of the 2 have been studied in 14 rabbits.

When doses totalling 20 c c to 30 c c were administered on the first, third and fifth days, precipitinogen disappeared from the circulation in an average time of 7.25 days after the last injection.

Precipitin production, with the same dosage, began invariably within 1 to 3 days, and reached its crest in an average time of 10.3 days. This time, as well as that required for the elimination of precipitinogen, is probably somewhat longer than the normal, for the reason that the frequent bleedings which were necessary in all likelihood altered the well-being of the experimental animals sufficiently to delay both processes to some degree.

After a period of from 30 to 50 days the antibody curve does not continue to pursue a steady downward path, but evidences a modified behavior: first, the curves representing the ring test and the flocculation test diverge markedly, the ring test curves persisting much longer than

¹⁸ Ztschr. f. Immunitätsf., 1909, 3, pp. 181 and 706.

the flocculation curves; second, soon after this period is entered on, flocculation ceases to occur in the lowest dilutions, although still continuing in higher dilutions; furthermore, the lower limit of flocculation rises with successive titrations, while the upper limit falls; therefore, curves representing the upper and lower limits of flocculation converge.

This is also characteristic of the ring test, but the altered behavior of the latter does not begin for some time after that of the flocculation test.

Three pairs of curves thus are shown in the graphs illustrating this later history of the antibody, representing the upper and lower limits of precipitation in the three following tests: (1) ring test, after 20 minutes; (2) flocculation test, after 2 hours; (3) flocculation test, after 18 hours, when precipitation is complete. The curves for the ring test persist long after both flocculation tests have become negative. The 2-hour flocculation curve ends soon after the period of modified behavior begins (within 5 to 20 days thereafter). The 18-hour curve continues 50 to 80 days after this period has begun. The ring test curve has not yet ended in any case studied, although one has been followed for 287 days after the beginning of this period.

INFLUENCE OF SATURATION ON PROPERTIES OF ANTIGEN IN THE WASSERMANN TEST

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The peculiar phenomenon which is exhibited by the alcohol-soluble and acetone-insoluble lipid obtained from various animal organs, in that it can play the rôle of antigen in the Wassermann test for syphilis, has been the subject of many investigations. The term antigen cannot be strictly applied to this lipid substance since it has been conclusively shown by Fitzgerald and Leathes¹ that repeated injections of this substance into rabbits do not lead to the appearance of antibodies in the blood stream. No adequate theory has been advanced which satisfactorily explains the mechanism of the reaction. It is unnecessary that the antigen be derived from the organs of syphilitic persons since the lipid may be, and is ordinarily, prepared from certain organs of normal animals. In fact, investigators have suggested the use of such substances as sodium oleate,² lecithin,³ and the bile salts,⁴ instead of the alcoholic extract of certain organs, but with the exception of cholesterol,⁵ which is at times added to the antigen, these have not come into general use.

The technic for the preparation of the antigen used in the Wassermann reaction has been standardized by Noguchi.⁶ It consists in the extraction of the macerated liver, heart or kidney of certain animals with absolute alcohol, evaporation of the extract, subsequent solution in ether and precipitation of the lipid by the addition of acetone. While this method usually yields potent preparations of antigen, it has nevertheless been noted that individual lots may at times exhibit marked antialexic [anticomplementary] properties. The antigenic value is, however, not materially affected.

Although the antigen appears to be quite universally distributed in the tissues, it occurs in the largest amount in the heart, liver and

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¹ Univ. Calif. Publications Pathol., 1912, 2, p. 39.

² Sachs, H., and Altman, K.: Berl. klin. Wehnschr., 1908, 45, p. 494.

³ Porges, O., and Meier, G.: Ibid., p. 731; Fleischman, P.: Ibid., p. 490.

⁴ Schürmann, W.: Med. Klin., 1909, 5, p. 627; Levaditi, C., and Yamansuchi, T.: Compt. rend Soc. biol., 1907, 63, p. 740.

⁵ Sachs, H., and Rondoni, P.: Ztschr. f. Immunitätsf., 1909, 1, p. 133; Browning, C. H.; Cruickshank, J., and McKenzie, I.: Biochem. Ztschr., 1910, 25, p. 85; Field, C. W.: Jour. Amer. Med. Assn., 1914, 62, p. 1620; Arch. Int. Med., 1914, 13, p. 790; Dreyer, G., and Ward, H. K.: Lancet, 1921, 1, p. 956.

⁶ Serum Diagnosis of Syphilis, 1912, p. 79.

kidney, which are known to contain highly unsaturated lipoids. These are exceedingly unstable and on exposure to the air are readily oxidized.⁷ The iodine value for freshly prepared lipid is usually over 100, while a short exposure to the air reduces this value to about 70. The possible effect of oxidation on the lipid was recognized by Fitzgerald and Leathes.¹ In order to avoid this factor they carried out their operations in an atmosphere of carbon dioxide, but they made no attempts to determine the influence of oxidation on the antigenic value of the lipid. In Noguchi's method for the preparation of the Wassermann antigen, no attempt is made to prevent oxidation, which undoubtedly takes place. Our work in this field has been limited to the determination of the effect of saturation of the lipid on its ability to act as the antigen in the Wassermann reaction.

The antigen which was used in these experiments was prepared by extracting finely ground fresh beef heart with 10 volumes of absolute alcohol for a period of 10 days. The solution was saturated with carbon dioxide to minimize oxidation. The supernatant fluid was removed by distilling in vacuo at a temperature below 37 C. The lipid residue was dissolved in ether, the solution was then poured into an excess of acetone, and the mixture was allowed to stand in the ice chest until the process of precipitation was complete. The supernatant fluid was decanted, and the acetone-insoluble precipitate was used for the subsequent experiments. About 0.3 gm. of this substance was dissolved in ether, and 9 c.c. of methyl alcohol were added to this solution. The mixture was filtered and preserved in a dark bottle. For the immunologic tests a 10% suspension, in salt solution, of the alcoholic solution was used as antigen. Despite the special precautions which were taken to prevent oxidation the iodine number of the lipid did not exceed 70.

Saturation of the lipid with hydrogen, whereby the double bond $-\overset{\text{H}}{\text{C}}=\overset{\text{H}}{\text{C}}-$ is reduced to a single bond $-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-$, was accomplished with the aid of the technic which has been suggested by Paal.⁸ About 200 mg. of the lipid were dissolved in absolute alcohol, moderate heat being used to affect solution. Fifty mg. of colloidal palladium, which were dissolved in a minimum quantity of water, were added to the alcoholic solution and the mixture was transferred to the shaking apparatus which has been described by Paal. After the air in the

⁷ Erlandsen, A.: *Ztschr. f. physiol. Chem.*, 1911, 51, p. 71.

⁸ *Ber. d. deutsche Chem. Geseltschs.*, 1902, 35, p. 2195; Paal, C., and Amberger, C.: *Ibid.*, 1904, 37, p. 124; 1905, 38, p. 1398; Paal, C., and Gerum, J.: *Ibid.*, 1908, 41, p. 805; Paal, C., and Oehme, H.: *Ibid.*, 1913, 46, p. 1297.

apparatus had been displaced by a stream of hydrogen, the outlet stopcock was closed and the inlet stopcock was connected with a gas buret which was filled with hydrogen, and the mercury level was so adjusted that the pressure of hydrogen in the system was about 10 cm. The hydrogenation apparatus was kept in motion by means of a small motor so that the maximum contact with hydrogen was secured, and the mixture was kept warm with the aid of a microburner. The hydrogenation was completed in about 30 hours. This was determined by noting that there was no further decrease in the volume of hydrogen. The product which was obtained from 5 such hydrogenation experiments was pooled, the alcohol was evaporated, and the residue was dissolved in anhydrous chloroform in order to free the lipid from the small amount of palladium and of contaminating protein. After evaporating the chloroform, the antigen was dissolved in methyl alcohol, and it was used in subsequent experiments in the same way as the unsaturated lipid preparation. An estimation of the iodine number of the saturated lipid gave a value close to zero, indicating that within the limits of experimental error hydrogenation had been practically complete. No indication that the solvents which were used in the preparation of the saturated lipid influenced its antigenic properties was obtained from a control experiment.

The well-known hemolytic system was used to determine the antigenic and the antialexic values of the saturated and the untreated lipid antigens. The antigens were used in 0.4, 0.3, 0.2 and 0.1 c c doses of 1:10, 1:50 and 1:250, etc., dilutions; doses of the other substances used are: 10% pooled alexin, 0.2 c c; inactivated human serum from which natural sensitizer had been removed by incubation with sheep cells, 0.2 c c; sensitized sheep cells, 0.2 c c; salt solution sufficient to bring the volume in each tube to 1.0 c c. The mixture of antigen, alexin and serum was placed in the ice chest over night and on the following morning after addition of the sensitized cells, the tubes were incubated at 37.5 C. until the control tubes to which no antigen had been added became clear. Table 1 shows the highest dilutions in which the antigens showed antialexic and antigenic properties.

TABLE 1
HIGHEST DILUTIONS IN WHICH ANTIGENS SHOWED ANTIALEXIC AND ANTIGENIC PROPERTIES

	Antigenic	Antialexic
Unsaturated antigen.....	1: 250	1: 20
*Control antigen.....	1: 250	1: 50
Saturated antigen.....	1: 1,250+	1: 830

* Unsaturated antigen treated with the same solvents as were used in the preparation of the saturated lipid.

In addition to the foregoing experiment, two human serums which were known to react triple positive and two which were negative were used in a test to check the value of the saturated lipoid as antigen in the Wassermann test. The antigens were used in one half of the minimum inhibitory dosage. The results are given in table 2.

TABLE 2
RESULTS OF EXPERIMENT WITH HUMAN SERUMS

	Dilution	Positive Serums	Negative Serums
Unsaturated antigen.....	1: 40	2	2
Control antigen.....	1: 100	2	2
Saturated antigen.....	1: 1,660	2	2

The results indicate that the saturated lipoid can be used as the antigen in the Wassermann reaction provided the dilution is sufficiently high to eliminate the antialexic effect. The two serums which were used were known to be triple positive; less positive serums were not available. It is possible that the saturated lipoid antigen cannot be used for as sharp a differentiation of weakly reactive serums as the antigen which is commonly employed.

A second series of experiments were carried out to determine the effect of light and of oxygen on the antigen and to check our first experiments with the saturated lipoid. A quantity of the lipoid which had been prepared according to the method of Noguchi was placed on a watch glass which in turn was placed in a desiccator which contained oxygen. The desiccator was then allowed to stand on the laboratory table so that a part of the day it stood in sunlight. A new lot of saturated antigen was prepared to act as a check on the previous work. The preparations were then tested for antialexic action with the following results:

TABLE 3
RESULT OF TESTING NEW LOT OF SATURATED ANTIGEN PREPARED AS CHECK ON PREVIOUS WORK

	Highest Dilution of Antigen Which Exhibited Antialexic Action
Hydrogenated antigen.....	1: 1,000
Antigen which was exposed to light.....	1: 80
Untreated antigen.....	1: 25

The effect of light and of oxygen is not marked, owing no doubt to the small amount of surface which was exposed to the oxygen. Two known triple positive syphilitic serums and one normal serum were

tested, using the foregoing antigen preparations in doses which correspond to one half of that which was found to be antialexic. The final result was the same in each instance. Our results appear to indicate that the ability of the alcohol- and ether-soluble and acetone-insoluble lipoid which is obtained from beef heart to act as antigen in the Wassermann reaction is not dependent on the degree of saturation since both the unsaturated and the hydrogenated preparations were found to be potent. The saturated lipoid appears to be distinctly more antialexic than the untreated antigen.

Noguchi and Bronfenbrenner⁹ compared the antigenic values of various acetone-insoluble lipoids with their iodine numbers and noted that all preparations with high antigenic values also possessed high iodine numbers, but this has not been corroborated by the experiments of Browning, Cruickshank and McKenzie.¹⁰ The former workers also noted that certain acetone-insoluble lipoids which possessed little antigenic value were also markedly antialexic. These results are difficult to interpret since the authors attempt to compare the antigenic properties of lipoids which were obtained from different sources with the corresponding iodine values. While we do not mean to imply that the degree of saturation is the only factor which may be concerned in antialexic action, it nevertheless appears to be one of the factors.

SUMMARY

Saturation of the lipoid commonly used as the antigen in the Wassermann test for syphilis was accomplished with the aid of hydrogen and colloidal palladium according to the method described by Paal. Both the unsaturated and the hydrogenated lipoid preparations are capable of acting as antigens in the Wassermann reaction. The hydrogenated lipoid preparation is distinctly more anti-alexix (anticomplementary) than the untreated antigen.

⁹ Jour. Exper. Med., 1911, 13, p. 43.

¹⁰ Ztschr. f. Immunitätsf., 1912, 14, 284.

STUDY OF AN ORGANISM RESEMBLING BACT. PULLORUM FROM UNABSORBED YOLK OF CHICKS "DEAD IN SHELL"

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In the spring of 1921, attention was called to the fact that a large percentage of eggs under artificial incubation at a local poultry plant failed to hatch, a condition known to exist generally in all establishments. From data gathered about several thousand eggs, it was learned that about 40% of the fertile eggs failed to hatch (table 1). The egg incubator with a capacity of 3,600 eggs was located in an exceptionally well ventilated basement, the temperature of the room being about 70 F. A new machine was installed for the 1922 hatch. The eggs were turned 3 times a day, and during this operation it is believed that they received sufficient aeration. It is apparent, then, that

TABLE 1
LOSSES IN ARTIFICIAL INCUBATION

Year	Fertile	Not Hatched	Hatched	Percentage Hatched
1919.....	5,384	3,752	1,632	30.3
1920.....	7,687	5,228	2,459	32.0
1921.....	3,832	1,768	2,064	53.9
1922.....	5,694	2,652	3,042	63.9
Total.....	22,597	12,800	9,797	43.3

as far as possible, optimum conditions were provided for the process of artificial incubation, and that the cause of the losses must be attributed to some factor other than poor management.

Examination of Table 1 discloses some interesting facts regarding the mortality of incubator chicks. The average hatch of all fertile eggs for the 4 years was 43.3%. The low percentage of hatch in 1919 and 1920 could probably be attributed to such factors as inbreeding, inter-current diseases and incubator trouble. The hatch for 1921 and 1922 may be considered as an average of what might be expected in most establishments.

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Pernot,¹ of the Oregon Station, made a bacteriologic study of chicks dead in the shell; he isolated an organism both from the heart blood and from the unabsorbed yolk. He further proved that the organism was pathogenic for chicks in the shell and also for young chicks shortly after hatching. Thinking, perhaps, that a similar condition existed in the plant under investigation, it was decided to make a bacteriologic study of the problem in much the same manner as that described by Pernot.

METHODS OF EXAMINATION

The eggs were brought to the laboratory as soon as possible after the death of the chicks had been determined, and the examination was made immediately. Three lots of 72, 36, and 36 eggs, respectively, were examined at different times.

A portion of the shell and membrane was carefully removed by means of sterile forceps, thus exposing the posterior end of the chick and the unabsorbed yolk. A red-hot spatula was used to sear an area over the unabsorbed yolk, after which a sterile loop was thrust into the yolk and a loopful removed and streaked over the entire surface of a meat infusion agar plate which was then incubated for 18 hours at 37 C. The reaction of the agar used was adjusted to about P_H 8.

With the aid of a binocular microscope, the plates were examined. A search was made for small, round, homogeneous colonies with irregular outlines, such as those described by Pernot. Colonies of this description were found on only 8 plates from the 72 eggs of the first lot examined; however, this type occurred in pure culture in every instance. Other plates showed staphylococci as a predominating type. Occasionally a gram-positive, spore-bearing rod was isolated, and in a few plates no growth took place.

The consistency of the yolk in all cases was much thinner than normal, and the blood vessels surrounding the sac were congested. In a few cases the yolk presented a reddish tinge, probably from hemorrhage into the sac. The latter change could not be correlated with the presence of any particular type of organism. Aside from this no other changes were noticeable. Failure to isolate an organism similar to that described by Pernot in a larger percentage of cases was thought to be due possibly to the small amount of material used in streaking the plates. In examining the second lot of 36 eggs, 2 loopful of material, instead of 1, were taken from each egg. Examination of these plates after incu-

¹ Oregon Agricultural Experiment Station Bull., 1908, No. 103.

bation showed the previously described type of colony occurring in pure culture in 9 instances. In examining the third lot of 36 eggs, 3 loopsful of material were used for streaking, but this did not increase the incidence of the previously described type of colony. In the plates from the second and third lots of eggs, staphylococci and gram-positive spore-bearing rods were also found.

It was noted that the organism was isolated from eggs in 2 instances from hens 2327 and 3848, and in 4 instances from eggs of hen 2248. This suggested the possibility of the hen as a carrier of the infection.

From the 3 lots of eggs, 26 strains were isolated which presented the same type of colony. A study was made of the morphology, cultural features and biochemic reactions of each strain, and all were found to be similar except in minor details which will be mentioned in the following description.

THE ORGANISMS

The organism in question was a short, gram-negative rod with rounded ends, and about the size of the white diarrhea organism. No spores or capsules could be demonstrated. They occurred both singly and in chains, some cultures containing quite long chains in which the elements could not be distinguished. While the size was rather constant, some variation in length occurred in a few strains. In a hanging drop the organism was found to be nonmotile.

CULTURAL FEATURES

When first cultivated the organism grew sparsely on the surface of an agar slant; the colonies remained isolated for several days, finally coalescing into a thin growth. However, after several generations, the growth was quite abundant at the end of 18 hours' incubation. In extract, or meat infusion broth, an even clouding occurred at the end of 18 hours' incubation. No pellicle was formed. A fine sediment collected after several days' incubation. No odor could be detected. Litmus milk remained unchanged for about 7 days, when an alkaline reaction could be detected. Coagulation and reduction began at the bottom of the tube and progressed upward until the whole became coagulated, and only a small ring at the top retained the intense blue color. Some strains required 60 days to complete this change. On agar plates the colonies appeared as very small, round colorless homogeneous specks. After several hours they became more opaque. There was a tendency of the colonies to remain isolated until after several generations. On potato, growth was very sparse even after several days'

incubation, though blackening of the potato began about the third day. When grown on Loeffler's coagulated horse serum, the colonies remained isolated and growth was not as abundant as on agar. In a gelatin stab, a moderate growth occurred along the line of puncture but no liquefaction of the mediums could be noted. On blood agar plates, made with defibrinated rabbit's blood, very fine colonies appeared along the line of streak, which finally enlarged and coalesced. No hemolysis of the cells occurred. On eosin-methylene blue plates, colonies did not appear until after 48 hours' incubation. They remained isolated and were dull blue.

BIOCHEMIC REACTIONS

The fermentation reactions of this organism are given in table 2.

TABLE 2
FERMENTATION REACTIONS OF THE ORGANISMS

	Acid	Gas
Dextrose.....	+	+
Mannite.....	+	+
Arabinose.....	+	+
Levulose.....	+	+
Rhamnose.....	+	+
Galactose.....	+	+
Xylose.....	+	+
Maltose.....	±	±
Dextrin.....	—	—
Dulcitol.....	—	—
Glycerol.....	—	—
Raffinose.....	—	—
Salicin.....	—	—
Inulin.....	—	—
Sol. starch.....	—	—
Saccharose.....	—	—
Lactose.....	—	—

+ indicates a positive reaction; — indicates a negative reaction; ± indicates an inconstant reaction.

The substance to be tested was placed in sugar-free meat infusion broth. The broth used was rendered sugar-free by inoculation with *B. saccharolyte* and by incubating for 48 hours. About 20 gm. of infusorial earth were added to each liter of medium and autoclaved for 30 minutes at 20 pounds' pressure. The broth was then filtered through paper, giving an exceptionally clear product. Twelve cc of a 0.2% alcoholic solution of brom-thymol-blue were added to each liter of broth and N/1 sodium hydroxide added until a grass-green color was obtained. Five cc were placed in Durham fermentation tubes and autoclaved for 30 minutes at 20 pounds' pressure. On inoculating a series of these tubes with *B. coli communior* no acid or gas was produced after several days' incubation, indicating that all fermentable substances had been removed by growth of *B. saccharolyte*.

Conductivity water was used in making the 10% solutions of the various fermentable substances. These were autoclaved for 15 minutes at 18 pounds' pressure. One-half of 1 cc of the solution was added to each fermentation tube. After the addition of the solutions, the tubes were incubated for 48 hours to detect contamination.

Every strain of the organism under study was inoculated into the various fermentation tubes at the same time and daily observations made over a period of 4 weeks. Acid was produced on xylose after 18 hours' incubation, though in the case of some strains gas formation was delayed several days. The same variation was noticed on levulose. On maltose inconstant reactions were found. Some strains produced acid and gas after 18 hours incubation, while others failed in this respect even after 4 weeks' incubation. One strain under study produced a bubble of gas in maltose on the third day of incubation; the gas was absorbed the 4th day and did not reappear until the 26th day. With the exception of the foregoing variations, all strains produced acid gas after 24 hours' incubation.

TABLE 3
COMPARISON OF THE ORGANISM UNDER STUDY WITH MEMBERS OF THE COLON-TYPHOID GROUP

Test Substance	Bact. from Eggs		Bact. paratyphoid A.		Bact. paratyphoid B.		Bact. pullorum		Bact. sanguinarium		Bact. anatum		Bact. enteritidis	
	A	G	A	G	A	G	A	G	A	G	A	G	A	G
Dextrose.....	+	+	+	+	+	+	+	+	+	—	+	+	+	+
Mannite.....	+	+	+	+	+	+	+	+	+	—	+	+	+	+
Arabinose.....	+	+	+	+	+	+	+	+	±	—	+	+	+	+
Levulose.....	+	+	+	+	+	+	+	+	+	—	+	+	+	+
Rhamnose....	+	+	+	+	+	..	+	+	±	—	+	+	+	..
Galactose.....	+	+	+	+	+	+	+	+	+	—	+	+	+	+
Xylose.....	+	+	—	—	+	+	±	0	±	—	±	±	+	+
Maltose.....	±	±	+	+	+	+	+	0	+	—	+	+	+	+
Dextrin.....	—	—	+	+	+	+	—	—	±	—	+	+	+	+
Dulcitol.....	—	—	+	+	+	+	—	—	—	—	+	+	+	+
Glycerol.....	—	—	±	—	±	—	—	—	—	—	±	—	+	—
Raffinose.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Salicin.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inulin.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sol. starch....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Saccharose....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lactose.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—

+ indicates positive reaction; — indicates negative reaction; ± indicates doubtful reaction; A indicates acid; G indicates gas.

From an examination of table 3, it is seen that the organism under study is differentiated from Bact. paratyphoid A and B, Bact. anatum and Bact. enteritidis by its lack of ability to produce acid and gas on dulcitol and dextrin; from B. sanguinarium by the fact that the latter organism never produced gas on any of the carbohydrates. Further examination of the data shows that it is almost impossible to differentiate the organism in question from B. pullorum by fermentation reactions, except that the former is apparently more constant in its fermentation of xylose.

All strains of the organism in question reduced nitrates to nitrites. The presence of nitrites was determined by the Griess method.

Each strain was grown in neutral meat infusion sugar-free broth for one week and then tested for indol production by using Ehrlich's aldehyde reagent; in no case was indol produced.

PATHOGENICITY

To test the pathogenicity of the organism under study 100 eggs were obtained from the poultry plant and incubated for 6 days. On the sixth day all infertile eggs were removed and the 66 fertile eggs remaining were used in testing the pathogenic powers of the organism. The tests were made by

TABLE 4
INFLUENCE ON HATCHABILITY OF INOCULATING EGGS WITH VARIOUS CULTURES

No. of Eggs	Injected with Strain	Dead	Hatched	Percentage Dead
1	40.....	1	0	100.00
5	29.....	5	0	100.00
4	1.....	4	0	100.00
3	37.....	3	0	100.00
3	23.....	3	0	100.00
4	30.....	4	0	100.00
4	41.....	4	0	100.00
3	401.....	3	0	100.00
4	M 2.....	4	0	100.00
4	371.....	4	0	100.00
3	11.....	3	0	100.00
2	8.....	1	1	50.00
2	39.....	2	0	100.00
1	48.....	1	0	100.00
1	32.....	1	0	100.00
1	4.....	1	0	100.00
1	34.....	1	0	100.00
1	292.....	1	0	100.00
1	5.....	1	0	100.00
1	33.....	4	0	100.00
1	293.....	1	0	100.00
1	6.....	1	0	100.00
1	7.....	1	0	100.00
1	341.....	1	0	100.00
1	402.....	1	0	100.00
1*	32.....	1	0	100.00
1	Bact. paratyphosus B.....	1	0	100.00
2	Bact. paratyphosus A.....	2	0	100.00
2	Bact. typhosus.....	2	0	100.00
6	Control.....	1	5	16.66

injecting 0.10 cc of a 24-hour broth culture into eggs containing developing germs. The side of the shell opposite the germ was disinfected with a 1:1000 solution of mercuric chloride, and with a sterile 22 gage needle a small hole was made through the shell and membrane through which the inoculations were made. Immediately after inoculation, the opening was sealed with a coat of paraffin and the eggs replaced in the incubator where they were turned 3 times each day throughout the incubation period. The pathogenic powers of every strain in question were studied by egg inoculation, a few eggs being inoculated with broth cultures of Bact. paratyphosus A, and B, and Bact. typhosus. Two eggs were inoculated with a culture of a staphylococcus, and 6 were left uninoculated as controls.

The results of these tests are given in table 4. The data recorded in table 4 show that death of the embryo occurred in all inoculated eggs except one of those inoculated with culture 8. This culture was

a staphylococcus thus showing that this organism may not be pathogenic; however, another egg inoculated with the same culture caused the death of the chick. All control eggs hatched except one. This egg was purposely selected as a control because in two instances eggs examined from this hen gave a culture of the type of organism under study. The data also show that such pathogens as Bact. paratyphosus A, B, and Bact. typhosus are also pathogenic for developing embryos. The hatching of one egg inoculated with a staphylococcus shows that in this instance the manipulations did not interfere with hatchability.

A bacteriologic examination was made of all unhatched eggs, and in every case an organism was isolated which corresponded to the type previously injected. A control egg which was not inoculated but failed to hatch also yielded an organism identical with the one under study.

TABLE 5
INFLUENCE ON HATCHABILITY OF INJECTING FERTILE EGGS WITH VARIOUS CULTURES AND STERILE SALT SOLUTION

Group	No. of Eggs	Injected with 0.1 c.c. of	No. of Eggs Hatched	No. Alive on 21st Day	Dead	Percentage Dead
1	27	Sterile salt solution.....	12	2	13	48.14
2	5	Broth culture Staph. aureus.....	1	1	3	60.00
3	5	Broth culture of unknown bacterium.....	0	1	4	80.00
4	5	Broth culture B. pullorum (recently isolated).....	0	0	5	100.00
5	4	Broth culture B. pullorum (laboratory strain).....	0	0	4	100.00
6	4	Broth culture of organism isolated from unabsorbed yolk of chick dead in shell.....	0	0	4	100.00
7	5	Controls.....	1	2	2	40.00

In view of the fact that an insufficient number of controls were used, it was decided to repeat the experiment in order to determine the effect, if any, of sterile salt solution on hatchability. Accordingly, a number of eggs were incubated, and on the seventh day, fertile eggs were selected and divided into 7 groups, which were treated as follows:

Group 1, consisting of 27 eggs, was injected with 0.10 c.c. of sterile salt solution.

Group 2, consisting of 5 eggs, was injected with 0.10 c.c. of a 24-hour broth culture of *Staphylococcus aureus*.

Group 3, consisting of 4 eggs, was injected with 0.10 c.c. of a 24-hour broth culture of an unknown bacterium of egg origin.

Group 4, consisting of 5 eggs, was inoculated with 0.10 c.c. of a 24-hour broth culture of *Bact. pullorum* which had been recently isolated from the heart blood of a chick.

Group 5, consisting of 4 eggs, was inoculated with 0.10 cc of a broth culture of an old laboratory strain of *Bact. pullorum*.

Group 6, consisting of 4 eggs, was inoculated with 0.10 cc of a 24-hour broth culture of an organism isolated from the unabsorbed yolk of a chick dead in the shell.

Group 7, consisting of 5 eggs, was not inoculated. All injections were made as previously described, and the eggs were incubated according to the usual method.

Table 5 gives the results obtained from this experiment.

An examination of table 5 shows that of the 27 eggs inoculated with sterile salt solution, 12 hatched and 2 chicks were alive but unable to get out of the shell on the 21st day. This indicates that the mechanical manipulations could not be responsible for death of the chick in all cases. In group 2, 1 chick hatched and 1 was alive and unable to extract itself from the shell on the 21st day, while 3 had died in the shell. From these results it is apparent that *Staphylococcus aureus* did not interfere with the hatchability of at least 1 egg. The 5 eggs in group 3, which had been inoculated with an unknown bacterium isolated from an egg, apparently killed the embryos except in one instance, and this chick was unable to extract itself from the shell on the 21st day. From the results obtained by the injection of eggs in groups 4, 5, and 6, it may be concluded that either an old or freshly isolated strain of *B. pullorum* may cause the death of the embryo. Also a strain isolated from the unabsorbed yolk of a chick dead in the shell may produce similar results. Of the 5 control eggs not inoculated, 1 hatched and 2 produced chicks which were unable to extract themselves from the shell on the 21st day of incubation. This indicates that some losses in incubation may be expected in normal eggs.

CONCLUSIONS

The losses in artificial incubation are exceedingly high.

An organism can be isolated from the unabsorbed yolk of some chicks "dead in the shell."

The constant finding of the organism in eggs from the same hen suggests the possibility that the hen may be a chronic carrier of the infection.

This organism is closely related to members of the colon-typhoid group and cannot be distinguished from *B. pullorum* by the fermentation reactions except by the inconstant reaction of the latter on maltose and xylose.

The organism appeared to be pathogenic for developing embryos.

Other members of the colon-typhoid group also appear to be pathogenic for developing embryos.

A strain of *Staphylococcus aureus* and an unknown bacterium isolated from eggs failed to cause the death of the embryo in all cases when injected into fertile eggs.

Fertile eggs inoculated with a small volume of sterile salt solution did not give an appreciably lower percentage of hatch than uninoculated eggs.

THE RELATIONSHIP OF THE ORANGE AND WHITE PYOGENIC STAPHYLOCOCCI

WITH SPECIAL REFERENCE TO VACCINE THERAPY

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The value of vaccine therapy in staphylococcus infections is now generally acknowledged. Most experienced workers deprecate the use of stock vaccines and claim that only autogenous vaccines yield good results. In Palestine, for reasons not quite clear, the number of staphylococcus infections are so numerous, and moreover the places where they occur are so remote from a laboratory, that the use of some form of stock vaccines is imperative. It was with the object of meeting this need that this study was undertaken.

Recent studies have brought to light the fact that among certain culturally identical pathogenic micro-organisms, immunologic types exist which are specific in their behavior. It was logical to assume that this condition prevailed also among the staphylococci and that once the serologic types were determined it would be easy to prepare an effective polyvalent stock vaccine from these type strains. Consequently, a number of strains of pyogenic staphylococci isolated from various conditions (furuncles, abscesses, mastitis, acne, etc.) were subjected to a detailed cultural and serologic examination. The results of this study are presented in this paper.

SOURCES OF STRAINS

In order to determine the prevailing types as they occur here normally in various infections, cultures were made from all the consecutive cases of staphylococcus infections that came to the laboratory for examination.

Thirty-five strains were tested, isolated from the following conditions: furuncles, 16; abscesses, 10; mastitis, 2; pyodermitis, 4; osteomyelitis, 1. The relation between source of culture and pigment production may be indicated as follows: The orange strains (26) were isolated from furuncles (11), abscesses (7), pyodermitis (2), eczema (2), and other pyogenic conditions—osteomyelitis, mastitis, ulcers and

pustules—(4) and the white strains (7) were obtained from facial furunculoses (5), puerperal sepsis (1) and mastitis (1). The orange types of staphylococci were, therefore, about 4 times as frequent as the white.

METHODS OF STUDY

The strains of staphylococci were tested culturally and serologically. Under the cultural tests were included pigment production, fermentation of glucose, mannite, lactose and saccharose and the liquefaction of gelatine. The serologic tests consisted of agglutination and of absorption of agglutinins.

CULTURAL PROPERTIES

The pigment production was noted in 48 hours of growth on agar slants. Fermentation of the sugars was tested on agar slants con-

TABLE 1
CORRELATION OF PIGMENT WITH CULTURAL REACTIONS

Pig- ment	No. of Strains	Dextrose		Lactose		Mannite		Saccharose		Gelatine	
		+	—	+	—	+	—	+	—	+	—
Orange..	26	26	0	25	1	25	1	26	0	22	4
White...	7	7	0	4	3	2	5	7	0	0	7

taining 1% of the respective sugar and Andrade's indicator. Gelatine liquefaction was studied by inoculating gelatine in Wassermann tubes, incubating at 37 C., for given periods, and then cooling the tubes and measuring the extent of the column liquefied. The results of the cultural tests are shown in table 1.

It is apparent from table 1 that on the basis of the cultural reactions alone it is difficult to make a clean-cut differentiation. All the orange and white strains ferment glucose and saccharose. A few of the strains failed to ferment mannite and lactose, and some did not liquefy gelatine. The orange staphylococci seem to be more homogeneous. The division of the cultures on the basis of pigment yields certain suggestive correlations.

It is seen from table 1 that all the white strains studied failed to liquefy gelatine, and most of them failed to ferment mannite. The orange cocci, on the other hand, actively liquefied gelatine and fermented mannite. The Winslows¹ and Kligler² reported marked quantitative

¹ Systematic Relationships of the Coccaceae, 1908.

² Jour. Infect. Dis., 1913, 12, p. 432.

differences in the rate of gelatine liquefaction between the orange and white cocci. In a later study of a larger number of strains Winslow, Rothberg, and Parsons³ found that this difference was not as marked as appeared from the earlier studies. They did find, however, that only 47% of their white strains liquefied gelatine as against 67% of their orange strains. It appears, therefore, that while there is a difference in the gelatine liquefying power of the orange and white strains, the difference is quantitative rather than qualitative. With regard to the sugar fermentations, there seems to be some disagreement. Winslow, Rothberg and Parsons,³ measuring the P_H in carbohydrate mediums report practically no fermentation of the mannite, while Dudgeon,⁴ using litmus as an indicator, reports active fermentation of this alcohol by both orange and white staphylococci. Our results on solid mediums with Andrade's indicator correspond more with those reported by

TABLE 2
AGGLUTINATION TESTS OF STAPHYLOCOCCI WITH VARIOUS SERUMS

Pigment	Number of Cultures	Positive Agglutination with Serums of			
		Aureus (No. 5) Maximum Dilution 1:160	Aureus (No. 9) Maximum Dilution 1:160	Albus (No. 18) Maximum Dilution 1:80	Albus (No. 4) Maximum Dilution 1:40
Aureus.....	26	24	13	2	9
Albus.....	7	0	1	4	5

Dudgeon. It is evident, however, that although quantitative cultural differences exist, it is not possible on the basis of these characters alone to differentiate the pathogenic staphylococci into distinct types.

SEROLOGIC TESTS

For the serologic tests rabbits were immunized with selected strains of orange and white staphylococci, respectively, and the agglutination and absorption tests made with these serums against all strains. The immunization was performed by injecting increasing doses of dead cultures on 3 successive days, followed by a rest period of 7 days and 3 successive injections of live cultures. Five to 7 days after the last injection the rabbits were bled. The titers of the serums varied from 80 to 160.

Table 2 shows the results of the agglutination of the various strains of staphylococci with serums produced with white and orange staphylo-

³ Jour. Bacteriol., 1920, 5, p. 145.

⁴ Jour. Path. & Bacteriol., 1908, 12, p. 242.

cocci. For clearness, the cultures are grouped on the basis of pigment. Controls uniformly failed to agglutinate. Each column is a summary of the number of orange and white strains agglutinated by the given serum in the maximum dilution. This summary is of interest in that it shows that there is a definite group relationship between the orange and white cocci, but that the aureus serums are more specific and do not agglutinate the albus strains to the extent which the serums of the latter agglutinate the aureus staphylococci. In either case, however, it seems to depend on the strain used in the production of the serum. Some strains (5 and 18) are apparently more true to type and produce only specific type agglutinins; that is, their serums agglutinate only

TABLE 3
ABSORPTION TESTS WITH DIFFERENT CULTURES AND SERUMS

Absorption by Culture	Minimum Dilution in which Agglutinins for Homologous Organisms Have Been Absorbed		
	Serum : Aureus, 5	Serum : Aureus, 9	Serum : Albus, 18
Aureus, 1.....	40	80	..
Aureus, 2.....	*
Aureus, 3.....	40
Aureus, 8.....	40	160	..
Aureus, 9.....	*
Aureus, 15.....	40
Aureus, 17.....	*
Aureus, 22.....	40	..	*
Aureus, 28.....	80
Aureus, 31.....	*
Albus, 4.....	..	160	80
Albus, 18.....	..	160	..
Albus, 27.....	..	160	*
Albus, 30.....	80
Albus, 33.....	*

* No absorption in highest dilution.

type strains; others (9 and 4), on the other hand, have a wider action; that is, their serums agglutinate orange as well as white cocci. In general, however, the results indicate that the orange and white cocci represent distinct but closely related varieties of the same species.

In order to ascertain more definitely the intra-and cross-relationship of the various strains, a series of absorption tests was made. The results of these tests (table 3) correspond with those of the agglutination tests and show that strain 5 is the type aureus strain, since all agglutinins are absorbed by other orange strains; strain 9 is less typical, since the group agglutinins it produces are absorbed by both orange and white strains while the specific agglutinins are left intact. Similarly, albus strain 18 resembles aureus strain 9 in that it produces both group and specific agglutinins, the latter not being absorbed by other white cocci.

The absorption tests bear out the conclusions based on the agglutination tests, namely, that the two pigment types are related. Both types of cocci produce group agglutinins for orange and white cocci, but there is a distinct specific difference between the two varieties—the orange variety being a more homogeneous and defined type. It is of interest in this connection that this type is encountered so much more frequently than are the white cocci.

PRACTICAL APPLICATION OF THESE FINDINGS

For practical purposes three stock vaccines were prepared: (1) an aureus vaccine consisting of strains 5 and 9, (2) an albus vaccine consisting of strains 18 and 33 and (3) a mixed albus and aureus vaccine consisting of all 4 strains.

In the laboratory, the following routine has been adopted: Polyvalent stock serums of strains 5 and 9 and of strains 18 and 33, respectively, are kept on hand, and each new culture is agglutinated with one or the other serum. If the agglutination is positive, stock vaccine is given, if negative, an autogenous vaccine is prepared. Since this procedure was adopted, more than 60 cultures of *Staphylococcus aureus* and 6 strains of *St. albus* were tested, and only 1 aureus and 1 albus culture failed to agglutinate. The strains selected represent, therefore, over 95% of the strains encountered in staphylococcus infections. The stock vaccines have also been tried on more than 50 cases, and from the clinical standpoint the results with the stock vaccines were as good as those obtained with autogenous vaccines.

For field purposes where making of cultures and typing are not possible, vaccines 1 and 3 are sent out. The aureus vaccine is tried first, and if the results are not favorable, the mixed vaccine is used. The vaccines have been used now in different parts of the country with entire satisfaction; the patients treated are reported to have responded favorably on the injection of the polyvalent stock vaccine.

SUMMARY

A systematic study was made of the immunologic types of orange and white staphylococci for the purpose of preparing a proper polyvalent stock vaccine. Two main types of aureus and 2 strains of albus were selected. The vaccines prepared with these strains were as effective therapeutically as were autogenous vaccines.

THE EFFECT OF THYROIDECTOMY, CONTROLLED BY RESPIRATORY EXCHANGE MEASUREMENTS, ON ANTIBODY FORMATION IN RABBITS

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The relation of the thyroid gland to the development of immune bodies has been studied many times on various animals, with widely different results. Some authors have reported significant increases, while others have reported no definite changes, or even decreases in antibody formation following thyroidectomy. The question, therefore, still remains unsettled.

Marbé¹ found a definite diminution of the opsonic index against staphylococci and tubercle bacilli in thyroidectomized dogs. Frouin² obtained a diminished production of hemolysin in thyroidectomized dogs. Fassin³ observed a marked diminution in the complement in thyroidectomized guinea-pigs. Fjeldstad⁴ was unable to obtain any appreciable effect on agglutinin (typhoid) formation in rabbits. Hektoen and Curtis⁵ did not find any noteworthy variation in the usual curve and amount of hemolysin formation in thyroidectomized dogs. Launoy and Lévy-Bruhl⁶ studied the effects of chicken spirochete infection on normal and thyroidectomized fowls. They were unable to demonstrate any difference in the resistance or any change in the intensity and rapidity of antibody formation. Lerda and Diez⁷ found that thyroidectomized guinea-pigs were equally or more resistant to tetanus and diphtheria toxins, strychnin and caffeine, than normal guinea-pigs. Other authors, Clevers,⁸ Houssay and Sordelli⁹ obtained an increase in agglutinin formation; the former worked with rabbits, the latter with horses. Houssay and Sordelli also found an increase in hemolysin formation in rabbits. Garibaldi,¹⁰ working with thyroidectomized rabbits, found unusually high hemolysin production. Houssay and Hug¹¹ obtained very high agglutinin titers in young horses. Ecker and Goldblatt¹² also obtained high hemolytic titers in thyroidectomized rabbits. Koopman¹³ studied hemolysin production in rabbits fed with desiccated thyroid, and noted very high titers. He was also able to increase the low titer of another animal by feeding with desiccated thyroid.

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¹ Compt. rend. Soc. biol., 1908, 64, p. 1058.

² Ibid., 1910, 69, p. 237.

³ Ibid., 1907, 62, p. 388; p. 467.

⁴ Am. Jour. Physiol., 1910, 26, p. 72.

⁵ Jour. Infect. Dis., 1915, 17, p. 409.

⁶ Compt. rend. Soc. biol., 1913, 75, p. 352.

⁷ Gior. d. r. Accad. di med. di Torino, 1905, 68, p. 195.

⁸ Compt. rend. Soc. de biol., 1921, 85, p. 659.

⁹ Ibid., p. 679.

¹⁰ Ibid., 1920, 83, p. 15.

¹¹ Rev. Inst. bacteriol. Dept. nac. hig., 1920, 2 p. 637.

¹² Jour. Exper. Med., 1921, 34, p. 275.

¹³ Endocrinol., 1919, 3, p. 318.

The aim of this work was to study the formation of immune bodies in an animal in which (1) thyroidectomy could be done without involving the parathyroid factor, and (2) in which the degree of thyroid insufficiency could be estimated by heat production measurements. The rabbit fortunately is well suited to meet both of these requirements. The parathyroids arising from the fourth gill arches are usually embedded in the thyroid lobes, and it is practically impossible to separate them from the thyroid gland. The parathyroids arising from the third gill arches are much larger than the fourth or internal parathyroids, and are usually so widely separated from the thyroid lobes that there is no danger of injuring their blood supply during thyroidectomy. Occasionally these also are attached to the thyroid lobes in which event they are injured in making the dissection. This occurred in two rabbits of this series; one developed tetany and died in spite of the administration of calcium, the other did not show any signs of tetany. Much of the work on dogs and guinea-pigs has been necessarily complicated by the parathyroid factor. The point of greatest importance in studies based on thyroidectomized animals, I believe, is to determine the degree of thyroid insufficiency produced. Accessory thyroids are common in all animals, and in the rabbit there is an additional factor—the difficulty of completely removing the main lobes. On account of their attachment to the larynx, often fragments, or it may be a few cells, are left behind, which can rapidly regenerate. A third factor, and probably the most important of all, is the control of iodine in any experiments in which the thyroid is concerned. Iodine, even for skin sterilization, should not be used. Very careful steps were taken in all the experiments here reported to exclude iodine, other than that naturally present in the food, and its possible effect on any remaining accessory or on unremoved thyroid fragment. The most striking effect of thyroid insufficiency is a reduction in the basal metabolism; this can be determined accurately and simply by measuring the heat production. Decrease in heat production in rabbits can be demonstrated usually between the sixth and eighth days after thyroidectomy and reaches its lowest level between the twentieth and thirtieth days. The drop in heat production shows wide variations, as would be expected, and in these experiments varied between 15 and 30%. After this, the animal either begins to compensate and the heat production rises, sometimes even reaching its previous normal level after a few months, or the low level of metabolism can persist for years, as demonstrated by Marine

and Baumann.¹⁴ Injections were begun 10 days after thyroidectomy in order to utilize the period of developing thyroid insufficiency for the study of antibody development, thus avoiding the stages of possible recovery or severe cachexia.

Healthy adult rabbits of about the same weight were used. Heat production measurements were made on all animals over a period of 2 weeks to establish normal averages of the metabolism. The Haldane apparatus was used. Then thyroidectomy with partial parathyroidectomy

TABLE 1
HEAT PRODUCTION MEASUREMENTS

Rabbits	Sex	Thyroid	Average Normal Metabolism Calories per Kg. per Hour	Average Metabolism End of First Week After Thyroidectomy	Average Metabolism End of Second Week After Thyroidectomy	Average Metabolism End of Third Week After Thyroidectomy	Percentile Drop After Thyroidectomy	Remarks
Series 1								
1.....	F	Removed	2.37	1.77	1.76	1.61	32.0	
2.....	F	Removed	2.21	1.97	1.95	1.94	12.2	
3.....	F	Removed	2.48	2.13	1.98	1.88	24.1	
4.....	M	Removed	2.29	2.01	2.00	1.85	19.2	
5.....	M	Removed	2.98	2.48	2.45	2.08	30.2	
6.....	M	Present	2.53	2.52	2.56	2.52	0.0	
7.....	F	Present	2.29	2.29	2.02	2.29	0.0	
8.....	M	Present	2.49	2.48	2.49	2.49	0.0	
Series 2								
9.....	M	Removed	3.21	2.29	2.29	2.23	30.5	
10.....	M	Removed	2.25	2.29	2.25	2.14	4.8	
11.....	M	Removed	2.46	1.94	1.72	1.57	36.1	
12.....	F	Present	2.47	2.42	2.43	2.45	0.0	
13.....	F	Present	2.42	2.39	2.39	2.41	0.0	
Series 3A								
14.....	M	Removed	2.88	2.40	2.26	2.26	21.5	Pneumonia
15.....	F	Removed	2.72	2.49	2.36	2.17	20.2	
16.....	F	Removed	2.37	1.83	1.79	1.67	29.5	
17.....	F	Removed	2.26	2.06	1.81	1.82	19.4	
18.....	F	Present	2.89	2.82	2.92	2.92	0.0	
19.....	F	Present	2.74	2.70	2.46	2.70	0.0	
20.....	F	Present	2.79	2.95	3.65	3.65	0.0	Pregnant

(internal parathyroids) was performed. Heat production measurements were continued during the entire course of the experiment. On the tenth day when the decrease in heat production was definite, the injections were started. A 50% suspension of washed sheep red cells was used. The same sheep was bled for all experiments; 2 c c, 3 c c and 5 c c of this sheep's cells' suspension were injected into the marginal ear vein every third day. Three days after the last injection the rabbits were bled for serum titration, and every third day thereafter for about 2 weeks. The natural sheep hemolysin was determined for each animal.

¹⁴ Am. Jour. Physiol., 1921, 57, p. 135.

Some of the animals gave slight hemolysis in 1:20 dilution; none of them showed hemolysis in 1:40 dilution. In all titrations 0.1 c c of 5% suspension of washed sheep cells, 2 units of complement and 0.05 c c of inactivated rabbit serum were used.

In the first series 8 rabbits were used, 3 controls, 5 thyroidectomized. The record of heat production shows a decrease in all thyroidectomized

TABLE 2
ANTIBODY TITRATIONS

Rabbits	Sex	Thyroid	Titrations			
			3 Days After Last Injection	6 Days After Last Injection	9 Days After Last Injection	12 Days After Last Injection
Series 1, Hemolysin						
1.....	F	Removed	500	700	600	
2.....	F	Removed	1,000	1,300	1,250	
3.....	F	Removed	700	1,000	800	
4.....	M	Removed	600	900	800	
5.....	M	Removed	500	900	700	
6.....	M	Present	600	800	700	
7.....	F	Present	1,200	1,100	1,000	
8.....	M	Present	1,000	1,100	900	
Series 2, Hemolysin						
9.....	M	Removed	800	1,200	1,400	1,200
10.....	M	Removed	1,100	1,250	1,250	1,100
11.....	M	Removed	800	1,200	1,500	1,300
12.....	F	Present	300	900	900	800
13.....	F	Present	850	1,200	1,100	
Series 3, Hemolysin						
14.....	M	Present	500	600	600	400
15.....	F	Present	1,100	1,200	1,400	1,100
16.....	F	Present	1,200	900	900	700
17.....	F	Present	900	1,100	900	
18.....	F	Present	700	900	1,400	1,200
19.....	F	Present	1,500	1,500	1,300	
20.....	F	Present	1,000	1,100	900	
Series 3-a, Hemolysin						
15.....	..	Removed	1,100	1,400	1,500	1,400
16.....	..	Removed	1,300	1,300	1,000	
17.....	..	Removed	900	1,500	1,500	1,400
18.....	..	Present	200	1,300	1,200	
19.....	..	Present	1,000	1,350	1,200	
20.....	..	Present	1,000	1,150	1,000	
Series 4, Agglutinin						
1.....	..	Removed	900	1,250	1,000	900
2.....	..	Removed	1,000	1,200	1,000	
3.....	..	Removed	900	1,000	800	
4.....	..	Removed	1,000	900	850	
7.....	..	Present	1,000	1,100	900	
8.....	..	Present	800	1,200	1,000	

rabbits, being on the average about 23.5% (numbers 1, 2, 3, 4, 5). No noteworthy change in the heat production of the control animals occurred (numbers 7, 6, 8).

In a second series, 5 rabbits were used. Two (numbers 12, 13) were kept as controls, and 3 (numbers 9, 10, 11) were thyroidectomized. Number 10 did not show any noteworthy change in heat production after thyroidectomy and cannot be taken as an animal with thyroid insufficiency. The other 2 rabbits showed a marked drop in heat production (table 1).

The titration curves show that some of the thyroidectomized animals (numbers 9, 11, 2) have a little higher titer than the controls, while in the others (numbers 4, 1, 3, 5) no noteworthy differences were obtained. The differences in the titers were so small that they can be considered as normal variations.

In a third series, the experiments were carried out in another way. Seven rabbits were used; heat production of all animals studied, natural hemolysin determined; none of the rabbits gave any hemolysis in a dilution of 1:20; then the injections of sheep cells were given as in the 2 previous series and the titrations made in the same way. The animals were left in the cages under the same conditions. After 8 weeks, blood was taken and the serums tested for hemolysin content, with negative results. Then rabbits 14, 15, 16 and 17 were thyroidectomized as in the previous series, and rabbits 18, 19 and 20 were kept as controls. Rabbit 14 died of pneumonia during the second week after thyroidectomy. Heat production measurements were again made and injections started on the tenth day after thyroidectomy. At that time all thyroidectomized rabbits showed a marked drop in heat production (table 1, series 3 a). Injections of sheep's cells, bleeding and titrations were carried out exactly as in the first and second series.

The titration curve showed that no striking change in the hemolytic titer occurred after thyroidectomy.

Antityphoid agglutinin formation was studied in the animals of the first series after the hemolysin studies had been completed. Polyvalent typhoid vaccine (2,500 millions in 1 c c) was used—0.25 c c, 0.5 c c, 0.5 c c and 1 c c were injected intravenously at 3-day intervals. Animals were bled for serum titrations on the 3d, 6th, 9th and 12th days after the last injection. The highest dilution of serum which gave complete agglutination with an 18-hour broth culture was taken as the titer. All titrations were made with the same stock typhoid culture. There was no noteworthy difference in the agglutination titer of thyroidectomized and control animals.

DISCUSSION

The method of injection used in the first series of experiments is open to objection because such large doses of sheep cells produce a shock from which the animals recover slowly. In the second and third series, the 5 c c dose of 50% sheep cells was divided into 2 parts and injected at intervals of 2 to 3 hours. In this way the shock may be avoided. As pointed out in the foregoing, thyroidectomy alone is not

sufficient evidence that the animal will develop thyroid insufficiency. This is illustrated in the variable percentile decreases in heat production, especially in rabbit 10, which showed only a slight decrease (4.8%) in heat production. Necropsy performed 2 months later revealed 5 small fragments of thyroid tissue, 2 of which it is believed were accessories. The high heat production in rabbit 20 was largely due to pregnancy.

Ten animals were inoculated with sheep cells during the period of most marked thyroid insufficiency as determined by heat production measurements, yet none of those animals developed hemolytic titers definitely higher than any of the nine controls.

CONCLUSION

The thyroid insufficiency, determined by heat production measurements, does not increase the formation of hemolysin and agglutinin (antityphoid) in rabbits.

Thyroid insufficiency does not inhibit the agglutinin and hemolysin formation in rabbits.

MICROSCOPIC DEMONSTRATION OF BACTERIA IN THE LESIONS OF EPIDEMIC (LETHARGIC) ENCEPHALITIS

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There are many facts which indicate a causal relationship between influenza (catarrhal fever mentioned by older writers), and diseases of the central nervous system. Acute encephalitis occurs during epidemics of influenza, and epidemics of lethargic and other forms of encephalitis usually follow widespread epidemics of influenza. The initial illness in many cases of encephalitis resembles influenzal infection. The significance of diplostreptococci of high virulence as a cause of influenza was amply demonstrated during the last pandemic. Diplostreptococci similar in morphology, but of low virulency as measured in the usual manner, have been isolated from various tissues in cases of epidemic encephalitis by many investigators. Von Wiesner¹ obtained a diplostreptococcus from the brain of a monkey that became lethargic following intracerebral inoculation of a brain emulsion from one of von Economo's² cases of encephalitis, and showed that these cultures of organism could be taken from the brains of persons that died of this disease.

Diplostreptococci or diplococci were isolated from the blood during the febrile stage in one or more cases of various forms of encephalitis by Nauwerck,³ by Pisani and Varisco,⁴ Oehmig,⁵ Boccolari,⁶ Orlandi,⁷ by Maggiora, Mantovani and Tombolato,⁸ and by Cohn and Lauber;⁹ from the spinal fluid by Brasher, Caldwell and Coombe,¹⁰ and by Tilney and Riley;¹¹ from the fluid of the lateral ventricle in 6 consecutive cases

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¹ Wien. klin. Wchnschr., 1917, 30, p. 935.

² Ibid., p. 581.

³ Deutsch. med. Wchnschr., 1895, 21, p. 393.

⁴ Riv. crit. di clin. med., 1920, 21, p. 217.

⁵ München. med. Wchnschr., 1920, 67, p. 660.

⁶ Policlin., 1920, 27, p. 122.

⁷ Riforma med., 1920, 36, p. 207.

⁸ Ibid., p. 114.

⁹ München. med. Wchnschr., 1920, 67, p. 688.

¹⁰ Brit. Med. Jour., 1919, p. 733.

¹¹ Neurol. Bull., 1919, 2, p. 106.

by Morse and Crump,¹² and in 1 case by Mathewson and Latham;¹³ from the deep portions of the brain of 1 case each by House¹⁴ and by Bernhardt and Simons,¹⁵ and from the brain and ventricular fluid in 5 of 15 cases of lethargic encephalitis by Siegmund.¹⁶ The results in animals were largely negative, but the agglutination experiments by Morse and Crump, and by Maggiora, Mantovani and Tombolato, and the immunization experiments by House indicate immunologic specificity of the respective strains isolated.

A diplostreptococcus was isolated by one of us (Rosenow¹⁷) from the brain of a case of acute encephalitis which occurred during the epidemic of poliomyelitis in 1916. The morphology and cultural characters were similar to those of the pleomorphic streptococcus isolated consistently from the brain and the cord of patients with poliomyelitis at that time. In 1918, a similar organism was demonstrated in the lesions of the brain and the cord of two children in the same family, who died with symptoms of acute encephalomyelitis.

Because of these and other similar findings, the scope of our study on the etiology of encephalitis, which has been in progress for the last three years, has been made to include the possibility that lethargic and other forms of encephalitis might be due to bacteria of ordinary morphology, but with peculiar neurotropic properties. The results from a study of only one case have thus far been published.¹⁷ In this case typical symptoms and lesions were produced in animals with a green-producing streptococcus isolated from infected teeth and tonsils, from the blood, and from the brain, and the organism was demonstrated in the lesions of the patient and of the experimental animals. An immune serum prepared with an identical streptococcus isolated from the tonsils of another typical case of lethargic encephalitis agglutinated this strain specifically, and afforded protection in mice against intraperitoneal injection.

We have examined sections of brain and medulla kindly sent us by pathologists from a series of cases of encephalitis which occurred in widely separated regions, and we shall report the methods used and the results obtained.

¹² Jour. Lab. and Clin. Med., 1920, 5, p. 275.

¹³ Med. Jour. Australia, 1917, 2, p. 352.

¹⁴ Jour. Am. Med. Assn., 1920, 74, p. 884.

¹⁵ Neurol. Centralbl., 1919, 38, p. 705.

¹⁶ Berl. klin. Wchnschr., 1920, 57, p. 509.

¹⁷ Jour. Am. Med. Assn., 1922, 79, p. 443.

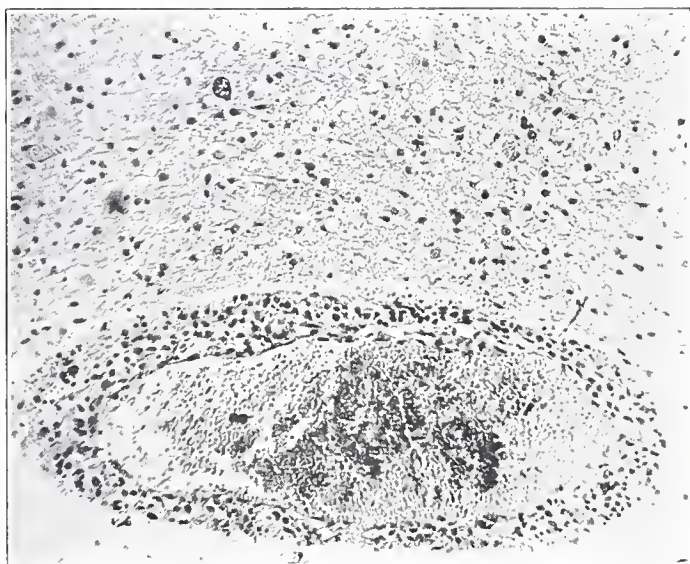


Figure 1 (Case 1)

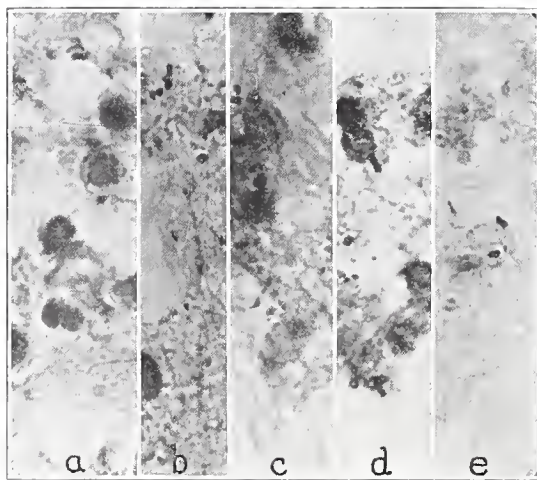


Figure 2 (Case 1)

The figures showing lesions in Cases 1, 2, and 3 were stained with hematoxylin and eosin (X 100). The figures showing bacteria were stained by the modified Gram method (X 1000).

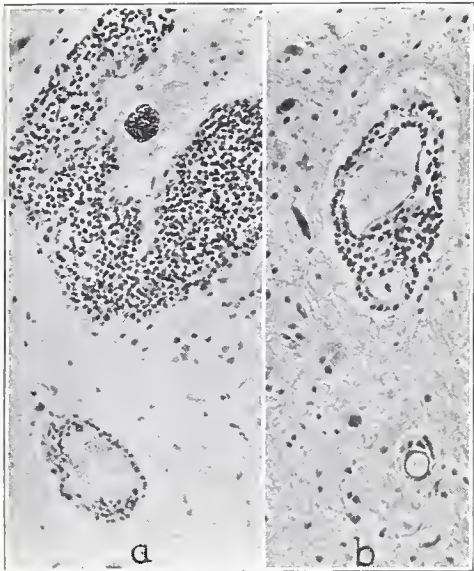


Figure 3 (Case 2)

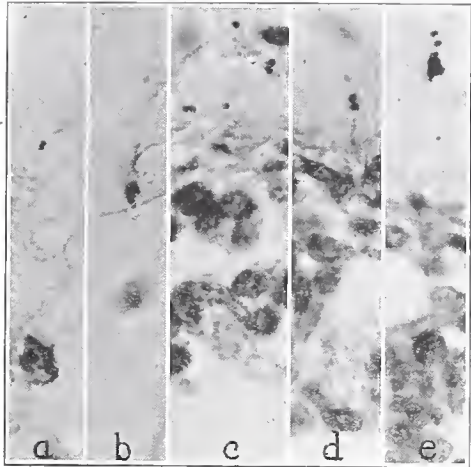


Figure 4 (Case 2)

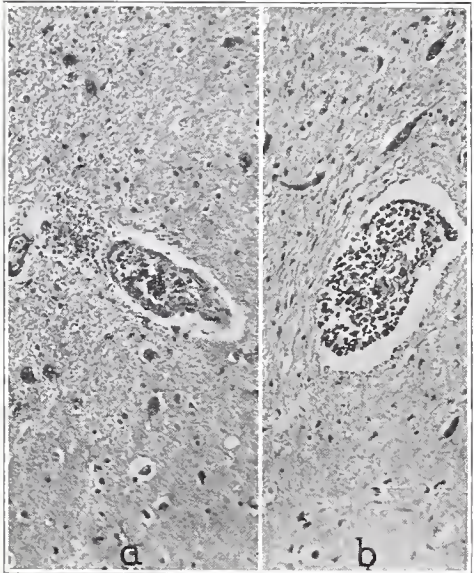


Figure 5 (Case 3)

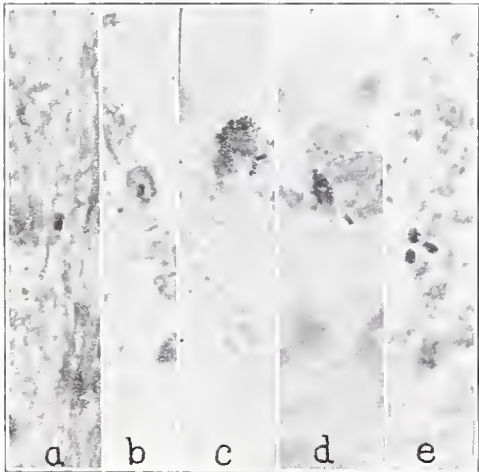


Figure 6 (Case 3)

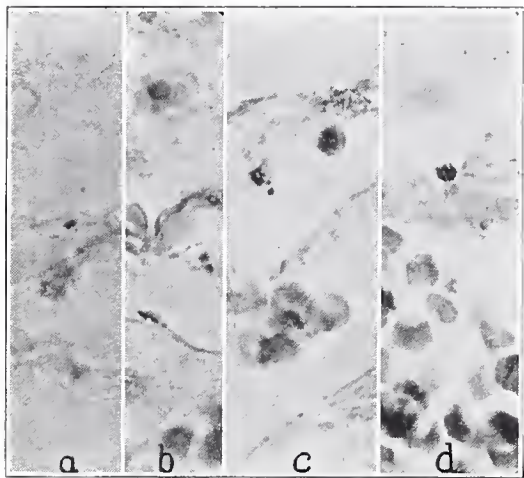


Fig. 7 (Case 4)

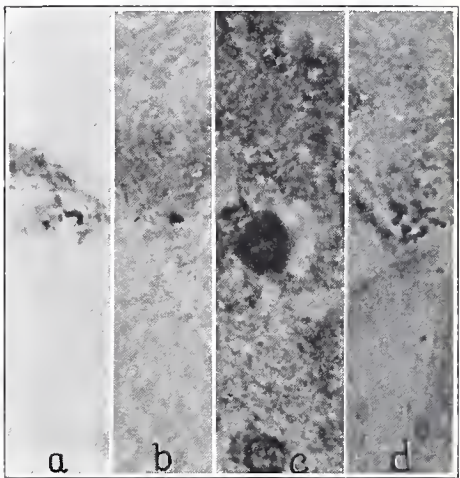


Fig. 8 (Cases 5, 6, 7, and 8)

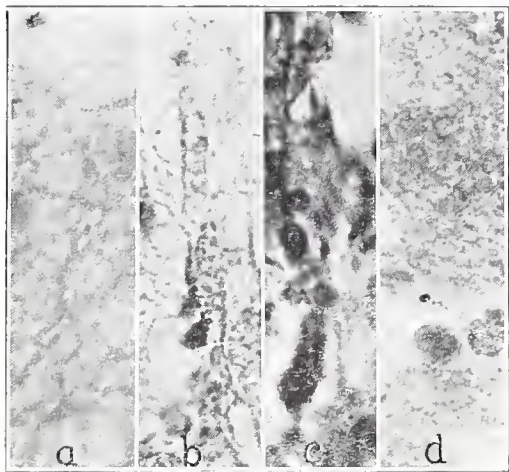


Fig. 9 (Cases 9 and 10)

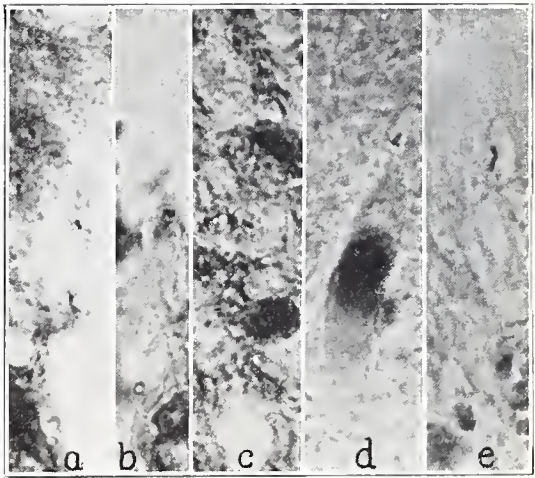


Fig. 10 (Cases 11, 12, 13, and 14)

The material studied was received in 10% formalin. Blocks were embedded in paraffin, and sections from 5 to 10 microns thick were stained for lesions with hematoxylin and eosin, and for micro-organisms by the Gram, the Gram-Weigert, and the Giemsa methods. In the Gram method, Kahlbaum's methyl violet was used instead of gentian violet, and decolorization was carried to a pale blue instead of to the end point. In this manner the nuclei stained light blue and the bacteria deep purple. Positive results were obtained with each of these methods, but the modified Gram method was the most satisfactory. The results obtained are summarized in the tabulation.

Material from 21 cases has been studied. The age of the patients ranged from 21 days to 69 years, the duration of symptoms from 24

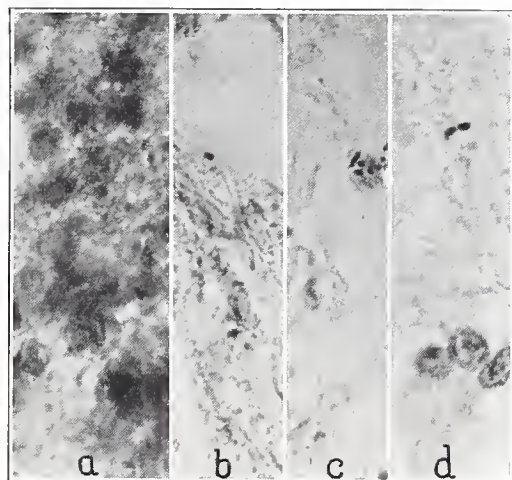


Fig. 11 (Cases 15, 16, and 17)

hours to 2 months. The lesions found were typical of encephalitis in all (figs. 1, 3, and 5), and undoubted cocci or diplococci, sometimes in short chains, were found in each of the 21 cases. Their demonstration was relatively easy in the material from cases that ran a short course, but difficult in those of longer duration, in some of which hours of search were necessary before bacteria were found. We soon learned that where one diplococcus was found, painstaking search often would reveal others in the same area. Typical diplococci, usually outside, more rarely within the cells (fig. 11 c), were found in the walls or just outside of blood vessels which were surrounded by edema, hemorrhage, or beginning round-cell infiltration (figs. 2 e, 4 b and c, and 7 b), in close proximity to dilated capillaries (figs. 7 a, 8 d, 9 b and 10 d), immediately adjacent

to perivascular and other infiltrates (figs. 2 a, 4 c and 8 c), in or adjacent to hemorrhagic areas in the gray matter of the pons and cerebrum (figs. 4 d and e, 6 e and 7 d), in or beneath the pia over the pons and medulla, and in sulci over the cerebrum (figs. 6 a, b, and c, 8 a, and 9 c). They were never found free in the lumen of vessels, nor in dense areas of perivascular round-cell infiltration, and rarely in normal tissue remote from lesions. No other bacteria were found in the 21 cases examined.

The size, shape and grouping of the organisms varied greatly. Large and small forms were often found in groups, more rarely in the same chain; and sometimes large oval or round forms similar to those found in old cultures appeared to be breaking into minute bodies (figs. 6 e, 7 c and d). Medium sized (figs. 2 b, 6 c, 8 a, 10 e and 11 d) and very small diplococci sometimes occurred in the same chain (figs. 2 e, 7 b and c). Exceedingly small cocci and diplococci, too small to photograph, were found in a number of cases. Some of the small forms were not unlike the filtrable bodies isolated from cases of acute epidemic encephalitis by Loewe and Strauss, by Thalhimer, by Maggiore and Sindoni, and by others, which they regard as the etiologic agent of the disease.

In order to check the efficiency of our staining methods, and the results obtained, we have examined sections of the brains of 20 patients who died of other causes. The sections were selected and stained in the same manner as those from the patients with encephalitis. In this control series were 2 cases of suicidal death by hanging, 3 cases of death from renal disease, 2 of carcinomatosis, 1 case each of extreme arteriosclerosis and cardiac insufficiency, and 1 of spinal cord glioma. In order to include conditions in which death occurred in connection with a process induced by bacteria, 3 cases of bronchopneumonia, 1 of diffuse tracheobronchitis, 2 of purulent meningitis, and 4 of tuberculous meningitis were also selected. In 13 of these 20 cases no bacteria were demonstrable. The 2 cases of purulent meningitis showed thickened and infiltrated meninges, short bacilli, and a few diplococci of uniform size. A few large deeply stained diplococci were found in the lumina of blood vessels or in tissue spaces free from lesions in 1 of the cases of carcinomatosis, in the case of diffuse tracheobronchitis, and in 1 of the cases of tuberculous meningitis, and an occasional diplococcus, with large gram-positive bacilli, in 1 of the cases of renal disease, and in 1 of the cases of tuberculous meningitis.

TABLE 1

RESULTS OF A STUDY OF MATERIAL FROM TWENTY-SIX CASES OF EPIDEMIC ENCEPHALITIS

Num- ber	Age, Yrs.	Dura- tion of Illness	Diagnosis	Microscopic Findings in Brain	Material Obtained from
1	6	10 days	Lethargic encephalitis	Marked perivascular infiltration in pons; medium-sized and very small diplococci in lesions, several short chains (figs. 1 and 2)	Dr. H. F. Robertson, University of Minne- sota, Minneapolis
2	18	10 days	Lethargic encephalitis	Congestion of vessels; adventitial and marked perivascular infiltration with round cells; moderate number of medium-sized diplococci in perivascular areas and in pia (figs. 3 and 4)	Dr. H. E. Robertson, University of Minne- sota, Minneapolis
3	34	4 weeks	Epidemic encephalitis	Perivascular edema, hemorrhage, and round-cell infiltration in pons; large, medium-sized, and small diplococci, and oval bodies breaking into minute forms (figs 5 and 6)	Dr. Peter Bassoe, Chicago
4	38	10 days	Lethargic encephalitis	Typical perivascular and other lesions; medium-sized and extremely small diplococci, and large bodies breaking into minute forms (fig. 7)	Dr. George W. Wheeler, New York Hospital, New York City
5	5	48 hours	Lethargic encephalitis	Slight round-cell infiltration surrounding vessels in pons and pia; a few diplococci, sometimes in chains, and large bodies breaking into small forms, in lesions (fig. 8a)	Dr. H. E. Robertson, University of Minne- sota, Minneapolis
6	43	5 weeks	Hemor- rhagic (lethargic) encepha- litis	Numerous areas of hemorrhage in pons, moderate neurophagocytosis, minimal perivascular infiltration; medium-sized diplococci in and adjacent to areas of hemorrhage (fig. 8b)	Dr. H. E. Robertson, University of Minne- sota, Minneapolis
7	44	7 weeks	Epidemic encephalitis	Marked perivascular infiltration in pons and medulla; small number of diplococci (fig. 8c)	Dr. Peter Bassoe, Chicago
8	Epidemic encephalitis	Typical lesions of encephalitis; diplococci singly, in short chains, and in groups in perivascular spaces (fig. 8d)	Dr. L. Hektoen, John McCormick Institute for Infectious Dis- eases, Chicago
9	18	2 weeks	Epidemic encephalitis (lethargic type)	Perivascular hemorrhage and round-cell infiltration in cerebrum and pons; medium-sized diplococci, sometimes in short chains, in lesions (fig. 9a and b)	Dr. W. Thalhimer, Columbia Hospital, Milwaukee
10	26	36 hours	Fulmi- nating epidemic encepha- litis	Marked perivascular round-cell infiltration of meninges over pons; slight infiltration otherwise; medium-sized diplococci in lesions (fig. 9c and d)	Dr. W. Thalhimer, Columbia Hospital, Milwaukee
11	37	24 hours	Fulmi- nating epidemic encepha- litis	Beginning perivascular infiltration by round cells; small areas of hemorrhage; diplococci readily demonstrable in perivascular spaces; no bacteria elsewhere (fig. 10a)	Dr. W. Thalhimer, Columbia Hospital, Milwaukee
12	16	4 to 6 weeks	Epidemic encephalitis; slowly pro- gressive type	Focal areas of round-cell infiltration; perivascular edema; a few diplococci in pia and pons (fig. 10b)	Dr. W. Thalhimer, Columbia Hospital, Milwaukee

TABLE 1

RESULTS OF A STUDY OF MATERIAL FROM TWENTY-SIX CASES OF EPIDEMIC ENCEPHALITIS

Number	Age, Yrs.	Duration of Illness	Diagnosis	Microscopic Findings in Brain	Material Obtained from
13	Epidemic encephalitis	Marked perivascular infiltration; moderate destruction and phagocytosis of ganglion cells; a few diplococci in pia and peripheral to areas of round-cell infiltration (fig. 10c)	Dr. G. B. Hassin, Cook County Hospital, Chicago
14	28	2 weeks	Lethargic encephalitis	Moderate degeneration of ganglion cells, neurophagocytosis, perivascular infiltration, and marked congestion; diplococci singly and in short chains adjacent to dilated capillaries and areas of infiltration (fig. 10d and e)	Dr. H. E. Robertson, University of Minnesota, Minneapolis
15	62	5 days	Epidemic encephalitis	Numerous petechial hemorrhages scattered throughout the cerebrum, with perivascular infiltration in midbrain; scattered diplococci of uniform size in areas of hemorrhage and just peripheral to perivascular infiltration (fig. 11a)	Dr. William Boyd, University of Manitoba, Winnipeg
16	35	2 weeks	Epidemic encephalitis	Characteristic vascular lesions of encephalitis; diplococci in and adjacent to areas of perivascular infiltration (fig. 11b)	Dr. William Boyd, University of Manitoba, Winnipeg
17	69	10 days	Epidemic encephalitis	Localized but slight perivascular infiltration, and hemorrhages in pons; diplococci in and adjacent to lesions in pia (fig. 11c and d)	Dr. J. S. McCartney, University of Minnesota, Minneapolis
18	53	2 months	Lethargic encephalitis; septic teeth	Localized adventitial and perivascular round-cell infiltration at base of brain; large and small cocci and diplococci in lesions	D. H. E. Robertson, Mayo Clinic, Rochester, Minnesota
19	Epidemic encephalitis	Marked changes; a few large and small diplococci in lesions	Dr. G. B. Hassin, Cook County Hospital, Chicago
20	Epidemic encephalitis	Marked perivascular infiltration in pons and medulla; few medium-sized diplococci in lesions	Dr. G. B. Hassin, Cook County Hospital, Chicago
21	Epidemic encephalitis	Perivascular edema and slight infiltration in pons and cerebrum; moderate number of medium-sized and small diplococci, one chain of 3 diplococci	Dr. G. B. Hassin, Cook County Hospital, Chicago

CONCLUSION

The presence of organisms in or adjacent to the lesions in a series of cases of encephalitis which occurred in widely separated communities, their absence in tissues free from changes, and in control sections from persons that died from other diseases, indicate causal relationship. The shape and grouping of, and the gradation between, large and small organisms, and the breaking of large forms into small bodies, indicate that the various forms are modifications of the same micro-organism.

FOOD ACCESSORY FACTORS (VITAMINS) IN BACTERIAL GROWTH

OBSERVATIONS ON THE ULTIMATE SOURCE OF ACCESSORY GROWTH SUBSTANCES FOR YEAST. VII

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It is generally conceded that animals are unable to synthesize vitamins from a vitamin-free diet, and it may also be true that the higher plants are devoid of this power, as maintained by Bottomley,¹ who believes that these substances are derived from the soil where they are generated by certain micro-organisms.

In searching for a possible ultimate origin of vitamins, it seemed appropriate to turn to the yeasts whose vitamin content has long been a subject of interest.

The work and subsequent controversies of Pasteur,² von Liebig,³ Mayer,⁴ and von Nägeli⁵ led to the belief that yeast grown in mediums whose only source of nitrogen was ammonium salts failed to give continuous growth. The addition of certain substances, such as yeast extract, uncleared beef heart extract, potato extract, carrot extract, etc., as growth stimulating substances has been reported by many observers. Wildiers⁶ termed these necessary substances "bios," and later work by Freedman and Funk⁷ indicated that the growth stimulating substance contained in a yeast extract is at least closely related to vitamin D. Funk and Dublin⁸ also concluded that vitamin D was a definite and specific substance stimulating the growth of yeast.

On the other hand, MacDonald and McCollum⁹ question the dependence of yeast on "bios" or a vitamin-like substance, while Nelson, Fulmer and Cissna¹⁰ concluded that yeasts can synthesize their own

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¹ Proc. Roy. Soc., B, 1915-17, 89, p. 102.

² Compt. rend Acad. d. sc., 1858, 47, p. 1011.

³ Ann. d. Chem., 1870, 153, p. 137.

⁴ Ann. Physik, 1871, 142, p. 293.

⁵ Chem. Zentralbl., 1881, 12, p. 45.

⁶ La Cellule, 1901, 18, p. 313.

⁷ Jour. Med. Res., 1922, 43, p. 457.

⁸ Jour. Biol. Chem., 1921, 48, p. 437.

⁹ Ibid., 1920-21, 45, p. 307.

¹⁰ Ibid., 1921, 46, p. 77.

vitamin B. Williams¹¹ maintains that water-soluble B is essential for the continued growth of yeast, and he has made this the basis of a test for this substance. The value and specificity of this test is still in doubt.

It is the purpose of the following experiments, not so much to establish the identity of a necessary growth stimulating substance, but to determine whether or not yeast is able to synthesize its own growth stimulating substance, be that what it may. In experiments with vitamins it is evident that special care should be taken in the use of chemically pure substances, controls, and the amount of yeast employed in the original as well as in subsequent inoculations.

The strain of yeast used in the following experiments was obtained from a cake of Fleischman's yeast, and furnished luxuriant growth on ordinary mediums.

The following medium was prepared, all substances being chemically pure: asparagin (Merck), 3.4 gm.; calcium chloride, 0.1 gm.; dextrose, 20.0 gm.; magnesium sulphate, 0.2 gm.; potassium phosphate (K_2HPO_4), 1.0 gm.; sodium chloride, 5.0 gm.; sterile distilled water to 1 liter.

These substances were dissolved by boiling for 3 minutes, the original volume restored with sterile distilled water, and the reaction adjusted to P_H 7.4. The medium was then tubed and autoclaved at 20 lbs. pressure for 30 min.

All yeast used to inoculate the tubes was grown in pure culture on dextrose agar slants, and thrown down and washed in sterile physiologic salt solution in the centrifuge 3 times before using. The inoculations were completed within 1 hour after centrifugation.

The following aqueous extracts were prepared:

1. Beef heart: Lean beef heart was finely ground, just covered with sterile distilled water, placed in the icebox for 18 hours and then passed through a Berkefeld filter.

2. Carrot: Finely grated carrot was covered with sterile water, allowed to stand at room temperature for 18 hours and then passed through a Berkefeld filter.

3. Potato: This was treated as carrot.

4. Yeast: This was grown on dextrose agar slants, suspended in sterile distilled water, and thrown down and washed with sterile distilled water 3 times in the centrifuge. The yeast was then killed by heating at 70 C. for 10 min. This suspension contained about 500,000

¹¹ Ibid., 1919, 38, p. 465.

cells per c mm. It was allowed to autolyze for 1 week at 37.5 C. and was then passed through a Berkefeld filter.

Tubes, each containing 2 c c of medium, were inoculated with the washed yeast suspension, a 3 mm loop being used uniformly. The tubes and results are shown in table 1.

TABLE 1

RESULTS OF INOCULATION OF TUBES OF MEDIUM WITH WASHED YEAST SUSPENSION

Medium	Growth
Synthetic medium.....	No continued growth
Sterile physiologic salt solution and beef heart extract.....	No continued growth
Sterile physiologic salt solution and carrot extract.....	No continued growth
Sterile physiologic salt solution and potato extract.....	No continued growth
Sterile physiologic salt solution and yeast extract.....	No continued growth
Synthetic medium and beef heart extract.....	Luxuriant growth
Synthetic medium and carrot extract.....	Luxuriant growth
Synthetic medium and potato extract.....	Luxuriant growth
Synthetic medium and yeast extract.....	Luxuriant growth
Dextrose broth (control).....	Luxuriant growth

All extracts in $\frac{1}{500}$ sol.

Growth was tested each 24 hours on dextrose agar slants using one loop. When the growth was positive transplants were made at the end of each 24 hours, thereafter using 1 loop of medium.

In all cases, positive growth was obtained for a few generations. That on the controls of salt solution plus the aqueous extracts continued only 2 or 3 generations while that on the synthetic medium was more prolonged, usually from 12 to 15 generations of growth being obtained, but diminishing slowly in luxuriance with each generation after the first 3 or 4 until it ceased entirely. Growth continued over a greater number of generations when transplants were made at the end of 7 days than when transplants were made every 48 hours. Reference will be made to this point later.

The comparative merits of the 4 aqueous extracts could not be judged as all produced a continuous luxuriant growth for a period of 50 generations when added to the synthetic medium in equal concentrations. It was found, however, that all produced the maximum growth when present in concentrations of about $\frac{1}{500}$ or higher. When present in concentrations of $\frac{1}{1000}$, the growth died out after a few generations, while concentrations greater than $\frac{1}{500}$ gave no greater luxuriance as judged by this rough evaluation.

It was observed that the duration and luxuriance of growth on medium which did not permit a continuous growth was to a certain extent directly proportional to the amount of yeast suspension used for

the original inoculation. No attempt was made to secure a numerical ratio, the observation simply being made that a 5 loop inoculation gave a growth several generations longer than a 1 loop inoculation, and that it in turn was in duration several generations less than the growth resulting from a 10 loop inoculation.

This may be satisfactorily explained by the observation that a suspension of washed yeast cells killed by heating at 70 C. for 10 min. in sterile physiologic salt solution P_H 7.4, permits the continuous growth of yeast, the original inoculation and growth tests being carried out as in the previous experiments.

It was found that within certain limits the luxuriance of growth thus obtained was directly proportional to the concentration of killed cells in the suspension, as shown in table 2.

TABLE 2
GROWTH OF YEAST AT VARIOUS CONCENTRATIONS

Concentration per C.mm.	Growth
25,000.....	++
50,000.....	++
100,000.....	+++
200,000.....	+++
400,000.....	+++

All concentrations, however, gave a continuous growth over a period of 50 generations, transplants being made every 48 hours.

If yeast be capable of synthesizing its own necessary growth substance or substances, it is evident that the inoculation of a small amount of vitamin-free medium with a small number of yeast cells should permit continuous growth, as should larger quantities of the same medium inoculated with a larger amount of yeast. In fact, to lessen the factor of autodigestion of which yeast is capable, as shown in table 2, it would appear essential that small quantities be used in all cases for the production of trustworthy results.

It was noted elsewhere that transplants made every 48 hours on the synthetic medium lessened the duration of growth as compared with transplants made every 7 days, but even when continued transplants were made after this period of time growth eventually ceased. It appears improbable that this difference of time is essential for synthesis of new growth stimulating substances by the yeast cells—the end-result being merely a prolongation of the period of growth for a not indefinite time—unless substances in the air play some as yet unknown part. It appears more probable that after some time the dead yeast cells were utilized

as food by the living, and that these growth stimulating substances of the older cells were in some way partially taken over in gradually diminishing amounts by the growing generation. A similar conclusion was arrived at by Eijkman, Van Hoogenhuijze and Derks.¹² Whether the ultimate cessation of growth occurs because of the extreme dilution to which these substances would be carried in a few generations, or because even at best this "taking over" is never complete, cannot now be stated with certainty. It appears most probable that both of these factors play a rôle, and this view is rendered more tenable both because these growth stimulating substances are required only in very minute amounts, and also because yeast cells with the addition of inorganic salts forms a suitable medium for the growth of yeast. Furthermore, this "taking over" apparently occurs both in the animal and vegetable kingdom, and is well demonstrated when man utilizes these products for food.

These factors doubtless played a part in the work of MacDonald and McCollum,⁹ who, using mediums in 25 c c lots, transplanted after 5-7 days with 1 c c of the agitated suspension and carried out their transplants for 15 generations with no apparent decrease in numbers.

Nelson, Fulmer and Cissna¹⁰ used 50 c c lots of medium and 1 c c for transplants, but eliminated to a large extent this "taking over" factor by transplanting every other day. They secured continuous growth for one year, and the feeding experiments performed by them on white rats with the yeast raised by this method constitutes a striking series of experiments. No mention was made by them, however, concerning the chemical purity of all the constituents used; and this fact, together with the large amount of yeast employed by them, would appear to render the results inconclusive.

SUMMARY

Yeast is incapable of synthesizing its own "growth stimulating" substance or substances on the synthetic mediums here described.

Beef heart, carrot, potato and yeast cells contain a water-soluble substance or substances which when added to this synthetic medium in high dilutions permit the luxurious and continued growth of yeast. In the concentrations in which these extracts were used they alone will not permit continued growth. These, or similar growth stimulating substances, seem therefore essential to the continued growth of yeast. Their ultimate origin is as yet unknown.

¹² J. Biol. Chem., 1922, 50, p. 311.

The luxuriance and duration of the growth of yeast on mediums which do not permit its continued growth is roughly proportional to the number of cells used for the original inoculation. It is also somewhat proportional to the length of time elapsing between the original inoculation and subsequent inoculations to successive transplants.

Yeast cells, to a large extent, possess the power of "taking over" these essential food substances when present in small amounts in the medium and utilizing them in further growth.

A suspension of killed, washed yeast cells plus the proper amount of inorganic salts forms a suitable medium for the continued growth of yeast.

STUDIES OF FUSIFORM BACILLI AND SPIROCHETES

II. THEIR OCCURRENCE IN NORMAL PREPUTIAL SECRETIONS AND IN EROSIVE AND GANGRENOUS BALANITIS

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Erosive and gangrenous balanitis was first recognized as an infection by Bataille and Berdal.¹ Their observations were confirmed by Scherber and Müller,² who emphasized the specificity of the infection in which fusiform bacilli and spirochetes were the causative factors. They further pointed out that the gangrenous was an advanced stage of the erosive form, both having a common etiology. Attention was directed to these infections in this country by Harris and Corbus,³ who found the same bacteria. They laid stress on such factors as contamination with saliva in persons with long foreskins, and they expressed the opinion that as the spirochetes were normally present in the preputial sac the fusiform bacillus was derived from some other source which ultimately related to the secretions of the mouth. Our observations began as a result of the occurrence of a case of gangrenous balanitis in which no history of contamination with saliva was obtainable.

A white laborer, single, aged 19, entered the Cook County Hospital, service of Dr. F. Phifer, with complaints of pain in the penis, cough, and pain in the chest. The swelling of the penis began 3 days previously at the middle of the shaft, associated with itching. The following day the swelling increased greatly, with marked pain. The cough, pain in the chest, and headache began 3 days prior to the development of the genital symptoms. The last sexual exposure occurred 6 weeks previously, and saliva contamination of any kind was denied. There was no previous venereal infection. On the day of admittance the temperature was 103 F., pulse 104. Physical examination was negative except for the genitalia, which presented great swelling and edema of the foreskin. From the preputial sac there exuded a brown purulent discharge. Phimosis was marked, and the foreskin could not be retracted over the glans. On the dorsum of the penis was a black area, 2 cm. long and 1 cm. wide, ulcerated and foul smelling. Under gas anesthesia a dorsal slit was performed, revealing a large amount of foul, brown, purulent material. The glans was eroded and ulcerated, and the entire prepuce was black. The gangrenous process spread rapidly, involving the entire shaft with a foul

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¹ Compt. rend. Soc. de Biol., 1889, 1, p. 689.

² Arch. f. Dermat. u. Syph., 1905, 77, p. 77.

³ Jour. Am. Med. Assn., 1909, 52, p. 1474.

discharge. The patient became irrational; he expectorated blood tinged sputum although no evidence of pneumonia appeared. After 5 days the pulmonary symptoms subsided, and the gangrenous parts began to slough, with final recovery. Direct smears stained with dilute carbol fuchsin revealed many typical fusiform bacilli, spirochetes and cocci. Aerobic cultures on blood agar gave many colonies of *Staphylococcus albus*. Anaerobic cultures in tall tubes of ascites dextrose broth and blood agar plates were foul-smelling and revealed staphylococci, together with short and long thread forms of *B. fusiformis*.

A second case of balanitis of the erosive type was then observed in a white laborer, suffering from pleurisy with effusion. During the

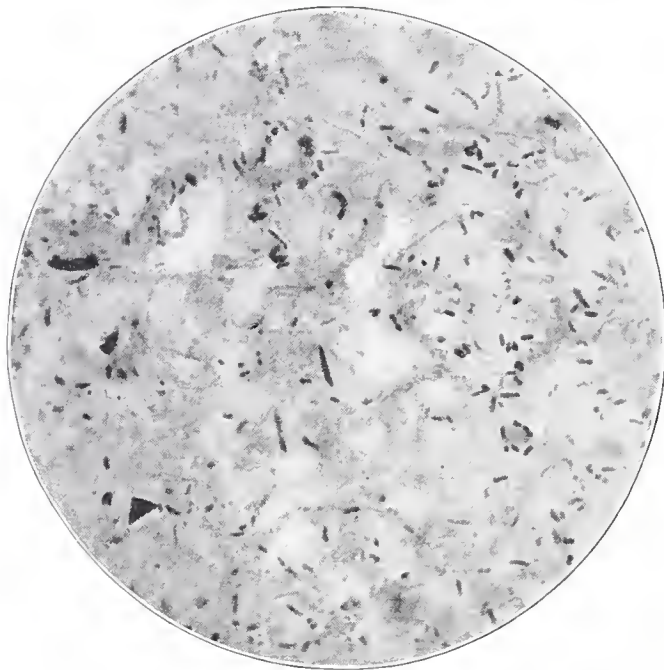


Fig. 1.—Smear preparation from erosive balanitis; X 1,000.

course of his pulmonary condition he developed swelling of the penis as the result of phimosis and neglect in the care of the preputial sac. Subsequently, 3 other cases of balanitis appeared; in one of these, contamination with saliva had occurred. In all of these the balanitis was of the erosive type from which the purulent secretions of the preputial sac revealed in direct smears fusiform bacilli, spirochetes and cocci (fig. 1). In the aerobic cultures on blood agar plates, *Staphylococcus albus* appeared in all, *Staphylococcus aureus* in 2 and *Streptococcus viridans* and *B. coli* each in 1. Anaerobically by the Dick plate method and tall tubes of ascites broth, foul smelling cultures were obtained in

which fusiform bacilli, often in thread forms, appeared, together with cocci, usually staphylococci. The most constant findings were the fusiform bacillus, spirochetes and cocci.

Particular emphasis is placed on the constant occurrence of pyogenic organisms, especially the cocci which are associated with the fusiform bacilli and spirochetes, since this is in accord with the constant presence of such cocci in the various infections of the mouth and lung due to *B. fusiformis* and spirochetes.⁴

Several instances of balanitis due to the fusiform bacillus and spirochetes have been noted in which, like our own, exposure or salivary contamination apparently were not essential factors. Owen and Martin⁵ reported 6 cases of balanitis with a history of saliva wetting in only 2, denial of such contamination in 3 and absolutely no exposure of any kind in 1. Sutton⁶ described gangrenous balanitis in a patient with no history of exposure. It is obvious that balanitis is by no means entirely dependent on the introduction of causative organisms through abnormal routes.

The absence of an adequate history of the source of infection led us to inquire into the normal flora of the preputial secretions. A review of the literature indicated that the observations were largely centered on the smegma bacillus which is nonpathogenic, and the smegma spirochetes, which were studied for differentiation from *Treponema pallidum*. Rona⁷ indicated the occurrence of bacilli and spirilla in cases of simple, erosive and gangrenous balanitis and demonstrated spirilla in few numbers, but no bacilli in the preputial secretions of 18 normal men. Schaudinn and Hoffman⁸ described a spirochete appearing about specific and nonspecific lesions of the genitals, differing from the spirochete of syphilis, and named it *Spirochaeta refringens*. Hoffman and Prowasek⁹ indicated some morphologic differences in the spirochetes of balanitis from the *Spirochaeta refringens*, and called the former *spirochaeta balanitidis*. Lowenberg¹⁰ also found spirochetes in the prepuce of normal persons and similar organisms in papillary condyloma, as well as ulcerative and gangrenous balanitis. Noguchi¹¹ succeeded in cultivating 3 different types of spirochetes from normal male smegma and named them *Spironema refringens*, *Treponema calligyrum* and *Treponema minutum*. Very little data could be found in connection with the occurrence of the fusiform bacillus which in other localities, as in the mouth, and in various infections is constantly associated with spirochetes. Rona⁷ observed bacilli and spirochetes in one case of flat condylomata. Donaila and Stroe¹² injected material from syphilitic lesions into the anterior chamber of a rabbit with resultant panophthalmitis, from the pus of which fusiform bacilli and spirochetes were identified. A systematic study of the possible occurrence of the fusiform bacillus about the normal genitals was not made by any of these workers.

⁴ Davis, D. J., and Pilot, I.: Jour. Am. Med. Assn., 1922, 79, p. 944.

⁵ J. Lab. & Clin. Med., 1917, 2, p. 862.

⁶ Jour Am. Med. Assn., 1918, 70, p. 675.

⁷ Arch. f. Dermat. u. Syph., 1903, 67, p. 259.

⁸ Berl. klin. Wchnschr., 1905, 13, p. 673.

⁹ Centralbl. f. Bakteriol., 1906, 1, O., 41, p. 741.

¹⁰ Dermat. Ztschr., 1911, 18, p. 27.

¹¹ Am. Jour. Syph., 1917, 1, p. 261.

¹² Compt. rend. soc. de Biol., 1913, 74, p. 298.

DIRECT SMEARS AND CULTURES FROM THE PREPUTIAL SAC

The subjects were men entering the Cook County Hospital and patients who had been in the surgical ward for several days. The men were chosen without discrimination, excepting only the circumcised. Care was exercised to exclude those who had or had had venereal infections. The total number examined was 100, 35 between 20 and 30 years of age, 65 between 30 and 40; 30% were colored. These patients represent the type that enter a charity hospital, coming largely from the poor classes.

The technic of obtaining smears consisted of retracting the foreskin, and with a cotton applicator collecting the smegma from the region just behind the glans, smearing the material on slides. In those cases in which there was some difficulty in obtaining secretion, a few particles were often found in the little pocket formed by the reflection of the frenum from the foreskin. The smears were stained with dilute carbol-fuchsin (10%) for 2 minutes. Of the 100 men thus studied, 51% harbored spirochetes, together with fusiform bacilli. Of the 30 colored, 12 or 40% were positive; of the 70 white, 41 or 59% were positive. The spirochetes (fig. 2) appeared often in large numbers, varying in size from 3 to 12 microns in length and from 0.2 to 0.5 microns in thickness. The undulations were irregular, varying with the character of the smear, and the ends pointed. Often two or three spirochetes appeared joined together end to end, in chain formation. The fusiform bacilli appeared usually in less numbers, from 3 to 7 microns long and from 0.5 to 1 micron wide, either straight, often curved slightly, tapering into sharp or blunt ends (fig. 3). Often the bacilli stained irregularly, appearing granular or striped. The bacilli were gram-positive if carefully decolorized, and the spirochetes were gram-negative. In all of the specimens, staphylococci, often diphtheroids and gram-negative bacilli and occasionally streptococci, were noted. An analysis of the results revealed that a smear having many spirochetes and fusiform bacilli was usually obtained from men with long phimotic foreskins, as a rule ill kept, with an accumulation of large amounts of moist secretion. The preputial sac in these instances apparently made an excellent culture medium for both aerobic and anaerobic organisms in symbiosis. The secretions in all cases had a characteristic slightly foul odor, which when marked, as in those with phimosis and considerable secretion, was rich in anaerobes, such as the spirochetes and fusiform bacilli.

For the identification of the fusiform bacillus anaerobic cultures were employed using the Krumwiede modification of the Dick plate

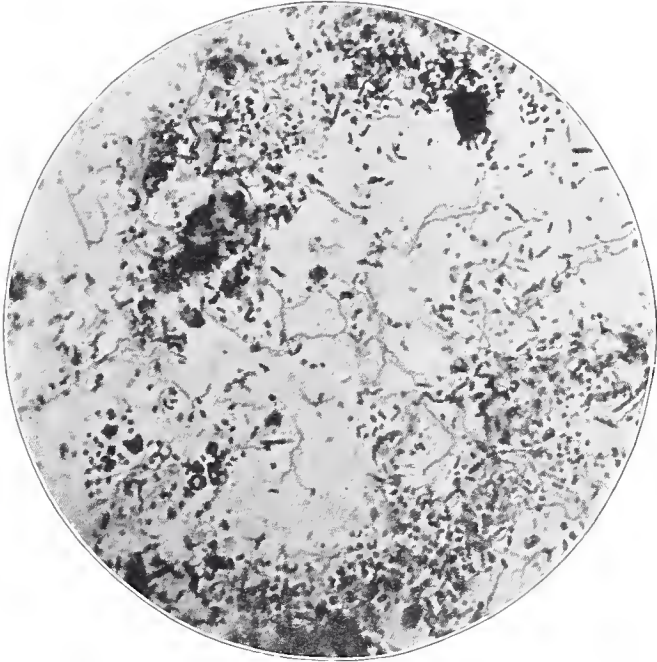


Fig. 2.—Smear of normal smegma revealing spirochetes, fusiform bacilli and cocci; X 1,000.

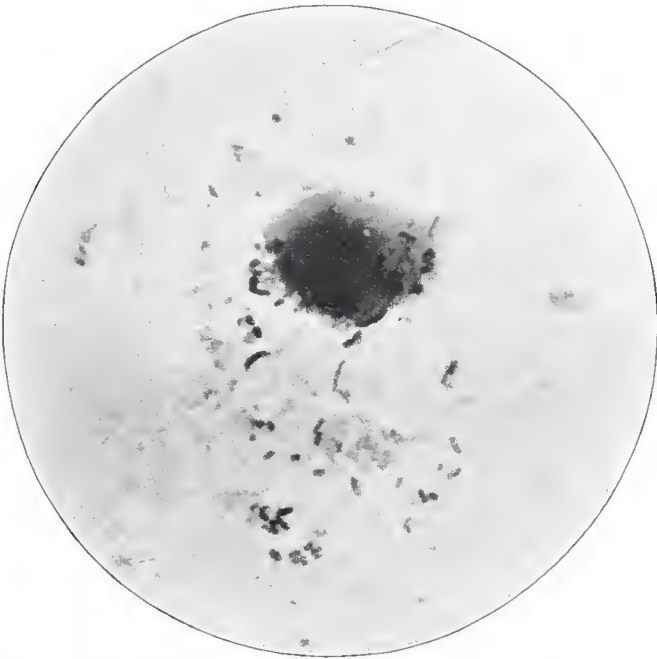


Fig. 3.—Smear of normal smegma illustrating straight and slightly curved forms of *B. fusiformis*; X 1,200.

method. In several heavy blood agar slants made anaerobic by the Wright method and tall tubes of ascites tissue broth, were also used. In the Dick plate the fusiform bacilli appeared as small irregular colonies, few in number, which in smears appeared as typical fusiform bacilli frequently curved and filamentous. In the ascites broth, fusiform bacilli grew chiefly as thread forms with cocci producing an intense putrid odor, especially about the tissue. Spirochetes were not observed in these cultures. In the Wright anaerobic slants, the fusiform bacilli occurred with many organisms, chiefly cocci, and the tubes had a putrid odor. The bacilli here were shorter, with tapering ends, sometimes accompanied by longer threadlike forms.

Aerobic cultures were made from the smegma of the 100 patients to determine the nature of the associated flora. Plain infusion agar, to which defibrinated human blood was added in the proportion of 1 part of blood and 9 parts of agar, was inoculated with preputial secretions collected in sterile salt solution. The poured plate method was used and readings made after 24 and 48 hours' incubation. The most constant organism found was the staphylococcus occurring in 90% of the specimens, usually in large numbers, of which 86% were of the albus and 4% of the aureus type. Fifty per cent. of the strains liquefied gelatin. Streptococci were found in 17%, usually in few numbers; 9% were of the hemolytic type and 8% of the viridans. Diphtheroids were quite prevalent, occurring in 50% and colon bacilli in 3%. Large gram-positive bacilli in colonies which often produced green discoloration of the blood agar were found in 28%. From these figures it is noteworthy that the preputial secretions harbor constantly large numbers of pyogenic organisms, particularly staphylococci and streptococci, which are constant factors in the production of all types of balanitis.

Animal Experiments.—To determine the pathogenicity of the bacteria of the preputial secretions, intrapleural injection of the material into rabbits was carried out in accordance with the method described in the production of putrid lesions from other sources.⁴ Normal preputial secretions from those individuals were injected into 3 different rabbits intrapleurally. One died in 48 hours, revealing purulent foul-smelling exudate in the pleural cavity. Cultures of the pus contained staphylococci, gram-negative bacilli and fusiform bacilli. The pericardial and peritoneal fluids yielded staphylococci and gram-negative bacilli, while the heart blood was sterile. A second rabbit was killed on the eighth day, and showed a foul exudate in the pleural cavity from which

staphylococci and fusiform bacilli were obtained in smears and cultures. The remaining fluids were sterile. The third rabbit was also killed on the eighth day. An organizing exudate was found from which staphylococci, fusiform bacilli and spirochetes in a few numbers appears in smears, and staphylococci and bacilli in the cultures. In a similar way a suspension of the purulent secretion from the necrotic tissue of the gangrenous mass from the patient with balanitis was injected intrapleurally into a rabbit. The animal was quite ill the next day and died within 48 hours. Necropsy showed a marked right-sided foul-smelling serofibrinous pleurisy. The pericardial cavity was hyperemic and contained a mucoserous exudate. Direct smears and anaerobic cultures from the pleural cavity revealed staphylococci and fusiform bacilli. The heart blood was sterile. From these experiments it is obvious that putrid lesions can be produced from both the normal secretions and the secretions of gangrenous balanitis.

From smears, cultures and experiments it appears that there is a striking similarity between the flora of normal preputial secretions and that of erosive and gangrenous balanitis. All revealed fusiform bacilli, spirochetes and cocci, the fusiform bacilli appearing in larger numbers in the pathologic secretions. We must recognize that, as in the mouth and in the tonsils,⁴ fusiform bacilli together with spirochetes are normally present as saprophytes in the preputial sac. In the mouth certain infections, such as noma, ulcerative gingivitis, etc., relate directly to the preexisting fusiform bacilli and spirochetes. In the same way, certain cases of simple, erosive and gangrenous balanitis arise from these organisms normally present. However, these infections result apparently only under certain conditions. A long foreskin with a tendency to phimosis and neglect in cleansing the sac leads to an accumulation of secretions. Often a lowered resistance coexists, such as that resulting from a general infection, undernourishment, or alcoholism. Then with such excellent anaerobic conditions as are found in the sac, the fusiform bacilli and spirochetes together with cocci produce the more advanced types of balanitis, varying from slight erosion to marked gangrene. In addition to their rôle as primary agents in balanitis, these bacteria may in some instances cause secondary infection in specific lesions. We have found them in chancroids, and they have been observed in papillomas.⁷ They are probably the organisms responsible for the marked ulcerations and gangrene that may complicate a primary syphilitic lesion on the penis.

SUMMARY

Under normal conditions fusiform bacilli and spirochetes were found in the preputial secretions of 51% of 100 men. As about the teeth and tonsils, these organisms exist as saprophytes in the preputial sac.

Associated with these bacteria were always pyogenic organisms, including staphylococci, streptococcus hemolyticus and viridans and colon bacilli.

A comparative study of the flora of the normal preputial secretions and the secretions from cases of erosive and gangrenous balanitis revealed a striking similarity in smears and cultures.

Of special interest is the fact that, with fusiform bacilli and spirochetes normally present in the preputial sac, balanitis is not entirely dependant on the introduction of these organisms from other sources but may arise locally, particularly in men with phimosis, retention of secretions and lowered general resistance.

SPECIFIC PRECIPITIN REACTION OF SEMEN

LUDVIG HEKTOEN AND LEONARD S. MANLY

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In 1901, C. G. Farnum¹ injected rabbits with semen in order to learn whether specific precipitins for semen would develop. This appears to be the earliest experiment of this sort. Farnum found that rabbits injected intraperitoneally with semen or testicular emulsions of dog, bull, or man developed antisemen precipitins. The antiserum for human semen had no action on human serum, indicating that the antisemen precipitin test might prove of practical value in studying the nature of seminal spots.

Strube² also obtained precipitins by injecting rabbits with human semen and testicular extracts, but these precipitins acted on blood serum as well as on semen, and he was not able to secure specific action on semen. H. Pfeiffer³ injected rabbits with dried and powdered bull spermatozoa, suspended in salt solution; the resulting antiserum acted strongly on semen solutions and testicular extracts and only feebly or not at all on extracts of other bovine organs; and by treatment of the antiserum with beef serum and certain organ extracts all precipitins except those specific for semen were removed. This treated antiserum caused precipitates in dilutions of bull semen and detected bull semen in mixtures with organ extracts.

No further experiments appear to have been made on antisemen precipitins until the work by Dervieux,⁴ who injected each rabbit with the semen of one person and claimed that he obtained a degree of individual specificness of reaction. His statement that precipitins for human blood do not act on human semen must be questioned. Uhlenhuth⁵ long ago found that the serum of rabbits injected with human blood proteins caused precipitates with human semen in low dilutions, which we also find to be the case.⁶

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¹ Jour. Am. Med. Assn., 1901, 37, p. 1721; Trans. Chicago Path. Soc., 1901, 5, p. 31.

² Deutsch. med. Wchnschr., 1902, 28, p. 425.

³ Wien. med. Wchnschr., 1905, 18, p. 637.

⁴ Compt. rend. d'Acad. d. Sc., 1921, 172, p. 1384.

⁵ Deutsch. med. Wchnschr., 1901, 27, p. 499; Wien. med. Wchnschr., 1904, 54, p. 2009.

⁶ For a comprehensive consideration of the immune reactions of the sexual cells, see W. P. Dunbar, Ztschr. f. Immunitätsf. u. exper. Therap., 1910, 4, p. 740; 1911, 7, p. 454.

We obtained semen from patients under the care of Dr. V. Lespinasse and Dr. R. D. Herrold who saved us specimens collected for clinical purposes. Four or 5 injections of mixtures of such samples were made intramuscularly in rabbits at intervals of 3 or 4 days, beginning with 2 cc and increasing the quantity by 2 cc each succeeding injection. As a rule, it was found best to bleed the rabbits for serum about 4 or 5 days after the last injection. As the antigen was not pure semen but mixtures of semen with inflammatory exudations and prostatic secretions, it was expected that the antisera would contain precipitins for human proteins generally, whatever the case might be as to precipitins for seminal proteins. The general results are illustrated in table 1 (serums 1-4), which shows that in rabbits injections of mixed human semen produced precipitins for human serum and for human semen, and that the precipitins for human serum may be removed by elective absorption, the rabbit serum now containing precipitins for human semen only. On the other hand treatment of the antiserum with semen dilutions removed all precipitins. In other words, the results show that a specific precipitin serum for human semen can be produced. In the earlier tests of antiserum, the clear fluids (*liquor seminis*) obtained by centrifugating samples of semen were used, and the figures in the table represent the highest dilutions of such seminal fluids and of human serum in salt solution giving positive results under the conditions of the tests. In order to remove the precipitins for human serum proteins, equal parts of antiserum and of dilutions of human serum 1 in 200 of salt solution are mixed, left at room temperature for about one hour and in the icebox over night, and then centrifugated thoroughly. Consequently "treated" antiserum is only half the strength of the original. As a rule, the procedure given removes all the precipitin for human serum; dilutions of about 1:200 give the best selective absorption. In the tests progressive dilutions of serum and seminal fluids are made in small, clean glass tubes, and the antiserum, original or "treated," is introduced at the bottom by small pipets so that a precise line of contact of the two fluids is obtained. The results are read after one hour at room temperature.

Table 1 also shows that practically identical results as those just outlined may be obtained from intravenous injections with extracts of spermatozoa (serum 5) in salt solution and of *liquor seminis* (serums 6 and 7). The extract of spermatozoa was made by adding large quantities of spermatozoa, already washed free from *liquor seminis*,

to salt solution, and shaking the mixture thoroughly. The clear fluid obtained on throwing down the spermatozoa by centrifugation is the extract. That spermatozoa continue to give up specific elements, peculiar to semen as well as to serum, on repeated washings may be determined by testing the corresponding extracts with precipitin serums. As shown in table 1, the serum of rabbit 5, injected with extract of spermatozoa, lost all its serum precipitin on standing, while the semen precipitin was retained in large degree. In testing the antisera of rabbits 5, 6, and 7,

TABLE 1

SPECIFIC PRECIPITINS FOR HUMAN SEMINAL PROTEINS IN SERUM OF RABBITS INJECTED WITH HUMAN SEMINAL PROTEINS

Serum of Rabbits Injected Intramuscularly with Human Semen (Serums 1, 2, 3, 4), Intra- venously with Extract of Human Spermatozoa (Serum 5), Liquor Seminis Humani (Serums 6 and 7)	Titers of Antisera in				
	Human Serum	Human Seminal Fluid	Extract of Human Spermatozoa in Salt Solution	Animal Seminal Fluids (Bull, Boar, Dog, Guinea-pig, Horse, Rabbit, Rat)	Salt Solu- tion
1. Original.....	6,400	800	0	0
Treated.....	0	256	0	0
2. Original.....	3,200	256	0	0
Treated.....	0	64	0	0
3. Original.....	6,400	640	0	0
Treated.....	0	256	0	0
4. Original.....	6,400	640	0	0
Treated.....	0	320	0	0
5. Original.....	800	320	0	0
After standing 7 months....	0	160	8,000	0	0
6. Original.....	1,280	1,280	16,000	0	0
Treated.....	0	320	8,000	0	0
7. Original.....	640	640	16,000	0	0
Treated.....	40	640	16,000	0	0
Normal rabbit serum.....	0	0	0	0	0

The figures give the highest dilution of serum, seminal fluid, and extract of spermatozoa in which the antisera caused distinct precipitates by the layer or contact method after one hour at room temperature. In the case of the extract of spermatozoa, the figures are based on the amount of protein in the solution as determined by dry weight.

table 1, we used an extract of spermatozoa containing 0.043 gm. of protein in each 100 c.c., and the figures in the table indicate that precipitates developed in dilutions of the extract up to 1 part of protein in 8,000 or 16,000 parts of fluid.

As stated elsewhere,⁷ a considerable number of tests of spots of human semen and other protein substances with antisemen serum, from which the precipitins for human serum proteins had been removed, gave results warranting the statement that antiserum for human semen may be of practical value in detecting by its specific precipitin reaction the presence of human seminal protein in suspected spots and stains.

⁷ Hektoen, Ludvig: Jour. Am. Med. Assn., 1922, 78, p. 704.

Experiments have been made also with swine semen, bull semen (Professor P. A. Fish, Cornell University), and equine semen (Professor W. S. Anderson, Kentucky Agricultural Experiment Station). These products, diluted with an equal amount of salt solution, were passed through Berkefeld filters, and the clear filtrates injected intra-

TABLE 2
SPECIFIC PRECIPITINS FOR BOAR SEMINAL FLUID

Serum of Rabbit Injected Intravenously with Filtered (Berkefeld) Boar Semen	Titers of Antiserum in			
	Swine Serum	Boar Seminal Fluid	Rat Seminal Fluid	Other Seminal Fluids (Bull, Dog, Guinea-Pig, Horse, Human, Rat, Rabbit)
Original.....	400	32,000	32	0
Treated with swine serum...	0	8,000	8	0
Normal rabbit serum.....	0	0	0	0

TABLE 3
SPECIFIC PRECIPITINS FOR EQUINE SEMINAL FLUID

Serum of Rabbit Injected Intravenously with Filtered (Berkefeld) Equine Semen	Titers of Antiserum in						
	Horse Serum	Horse Seminal Fluid	Boar Seminal Fluid	Bull Seminal Fluid	Dog, Guinea- Pig and Rat Seminal Fluid	Human Seminal Fluid	Rabbit Seminal Fluid
Original.....	100	5,000	20	40	0	40	20
Treated with horse serum.....	10	2,500	0	0	0	0	0
Normal rabbit serum...	0	0	0	0	0	0	0

TABLE 4
SPECIFIC PRECIPITINS FOR BOVINE SEMINAL FLUID

Serum of Rabbit Injected Intravenously with Filtered (Berkefeld) Bovine Semen	Titers of Antiserum in		
	Bovine Serum	Bull Seminal Fluid	Boar, Dog, Guinea-Pig, Horse, Human, Rabbit and Rat Seminal Fluids
Original.....	0	1,280	0
Normal rabbit serum.....	0	0	0

venously in rabbits at 3-day intervals in quantities of 1, 2, 3, 5, and 7 c c, in the case of swine and horse semen, and in quantities of 2, 4, 6, 8, and 10 c c in the case of bull semen. In all instances, the serum was found to have a satisfactory precipitin content on the fourth day after the last injection.

The results are illustrated in tables 2; 3, and 4, in which the figures give the highest dilutions in which precipitates formed at the place of contact after one hour at room temperature. The species and semen specificness of the reactions is striking. In the case of the antisera for swine and equine semens specific absorption with serum dilutions 1:200 removed practically all precipitins except those for the homologous seminal fluids, while the bull antiserum was limited originally to bull seminal fluid (table 4).

SUMMARY

The injection of rabbits with human semen, with human seminal fluid, or with extract of human spermatozoa induces the formation of precipitins that are specific for human seminal proteins.

The seminal precipitin reaction promises to be of value in determining the nature of suspected seminal spots and stains.

Swine, bovine and equine seminal fluids, injected in rabbits, also lead to the production of species and semen specific precipitins.

Serum precipitins in antiserum for semen may be removed by selective absorption with the proper serum in a 1:200 dilution.

The precipitin reaction may be of value in the study of the constituents of the sex cells.

INCIDENCE OF HEMOLYTIC STREPTOCOCCI IN NORMAL PREPUTIAL SECRETIONS OF MEN

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In bacteriologic studies of the secretions of the preputial sac of men, we have observed the occasional occurrence of hemolytic streptococci. A study was made of these streptococci as a part of the investigations that have been carried on in our laboratory in the distribution and normal habitat of these organisms.¹

The material was collected from the preputial sac of 100 normal men from 20 to 40 years of age, who were free from genital lesions. The patients entered the Cook County Hospital examining room, and were from the poorer walks of life. The secretions were suspended in sterile salt solution, and cultures were made by inoculating blood agar consisting of 1 part of human defibrinated blood and 9 parts of infusion agar titrated to a hydrogen-ion concentration of 7.6. The medium was inoculated in the melted state at 45 C. and poured into plates. The plates were incubated at 37.5 C. and examined at the end of 24 and 48 hours for hemolytic colonies.

Three types of hemolytic colonies were encountered. Large white or orange colonies with wide zones of hemolysis proved to be staphylococci. Smaller gray colonies often were hemolytic colon bacilli. Cocci forming small discrete gray-white colonies with zones of complete hemolysis measuring 2 to 4 mm. were isolated in pure culture for further study and confirmation.

Hemolytic streptococci were found in 9 of 100 specimens of secretion. They numbered always far less than 10% of the total number of colonies on the plate. In several instances, only 2 or 3 colonies of streptococci were present.

In the subcultures, the colonies were typical, growing as small round biconvex colonies with a zone of clear hemolysis measuring 2 to 4 mm. across. They corresponded in their appearance to the beta type of hemolytic streptococcus. In smears they occurred as gram-positive round cocci in chain formation. Litmus milk was acidified in all

Received for publication, Dec. 12, 1922.

¹ Davis, D. J.: *The Habitat and Distribution of Dangerous Hemolytic Streptococci in the Body*, Ill. Med. Jour., 1919, 36, p. 134.

instances and coagulated in two. The remainder coagulated on gentle heating; in 1% carbohydrate broth with Andrade indicator inoculated with the strains, flocculent sediment formed, while the supernatant fluid remained clear. All fermented lactose and salicin, none fermented mannite and inulin. In accordance with Holman's classification, these streptococci would fall in the group of *Streptococcus pyogenes*.

In their pathogenicity for rabbits they seemed to be somewhat inferior to strains of *Streptococcus pyogenes* isolated from the tonsils² and adenoids.³ Suspensions of growth of one blood agar slant injected intravenously produced only slight seropurulent arthritis; 3 c.c. of an ascites broth culture produced a more marked arthritis but did not kill the animals. Three strains were tested thus.

Other streptococci encountered were of the viridans type which occurred in few numbers in 8%. These strains all fermented lactose; all but 2 fermented salicin, and none fermented mannite or inulin. They appeared to be similar in their cultural and fermentative properties to the *Streptococcus mitis* and *salvarius* that are found about normal teeth and tonsils.

The staphylococcus, chiefly of the albus type, appeared in large numbers in 90% and is to be considered as the most constant normal inhabitant of the sac. Diphtheroids, colon bacilli and others were found less constantly and in fewer numbers.

From our studies, it is evident that in a few persons the preputial sac may harbor small numbers of streptococci. The men that were selected were of the type whose hygiene of the sac and bathing were often neglected. Undoubtedly men of better habits would reveal less of these organisms. Schacter⁴ observed that of 38 persons accustomed to frequent bathing, none showed streptococci in cultures of the skin; but of 89 dispensary patients, there were hemolytic streptococci in 8, especially in cultures made of hairy parts. The preputial sac and moist hairy parts, therefore, appear to be regions in which these bacteria may occur. The possibility should be considered that under certain conditions they may give rise to inflammation of the genitals, acting together with the fusiform bacilli and spirochetes in the production of balanitis. They may, too, be a source from which streptococcal infections of the penis and scrotum may arise, as reported by Campbell.⁵

² Pilot and Davis: *J. Infect. Dis.*, 1919, 24, p. 386.

³ Pilot and Perlman: *Ibid.*, 1921, 29, p. 47.

⁴ *Trans. Chicago Path. Soc.*, 1918, 10, p. 301.

⁵ *Surg., Gynec. & Obst.*, 1922, 34, p. 780.

In analyzing the data now available as to the distribution of hemolytic streptococci in normal persons, we must recognize that their occurrence in regions other than the mouth is unusual, and that the most constant habitat is the oro- and naso-pharynx, particularly in the crypts of the tonsils.^{2, 3}

SUMMARY

Hemolytic streptococci were isolated and identified in the preputial secretions of 9 of 100 normal men. They always occurred in few numbers. These streptococci agree in their morphology, cultural characteristics and fermentations reactions with the *Streptococcus pyogenes*. They appeared to be somewhat less pathogenic than similar streptococci from tonsils and adenoids.

The occurrence of the streptococcus in preputial sacs generally is presumably uncommon, particularly as the men here studied were careless about the hygiene of the sac. The staphylococcus appears to be constant in large numbers. The sac resembles in its coccal flora the moist hairy parts of the body.

FACTORS CONTROLLING INTESTINAL BACTERIA

THE INFLUENCE OF HYDROGEN-ION CONCENTRATION ON BACTERIAL TYPES

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The transformation of the intestinal flora depends on a number of factors, such as diet,¹ degree of intestinal absorption,² rate of intestinal secretion, and conditions of motility. Antibody mechanisms,³ hydrogen-ion concentration, and other factors⁴ may also have a part in determining the relative proportions of various bacterial groups. However, under normal physiologic conditions, it appears safe to conclude that the bacterial types are mainly influenced by the chemical nature of the ingested food. The explanation of this effect of diet on the intestinal flora is still an open question, and the various interpretations of the facts indicate the need of further investigation. Workers in this field appear to agree that an animal protein diet encourages the growth of proteolytic types, whereas the addition of lactose or dextrin stimulates the development of aciduric micro-organisms. Normally, a sufficient amount of lactose in most instances will cause a complete transformation of an ordinary mixed flora into one consisting predominantly of streptococcal and lactobacillary types.

There are two views in explanation of these facts. One is that the carbohydrate offers a readily utilizable source of energy for both aciduric and proteolytic types, and as a result fermentation instead of proteolysis occurs. At the same time, acid is produced which tends to create an environment unfavorable for the proteolytic types, and which may eventually lead to their elimination. The other view is that the carbohydrate simply insures an optimum cultural environment for the aciduric organisms, but that acidity plays no part in the elimination of the proteolytic types. The work described in this paper was done with the object of getting more facts in the matter, particularly with reference to the relationship of the hydrogen-ion concentration to the various bacterial groups.

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¹ Rettger and Cheplin: The Transformation of the Intestinal Flora, 1921.

² Dragstedt, Cannon and Dragstedt: Jour. Infect. Dis., 1922, 31, p. 209.

³ Torrey and Rahe: Jour. Immunol., 1920, 5, p. 133.

⁴ Eiskamp and Park: Jour. Infect. Dis., 1921, 28, p. 67; Jordon, E. O.; Abstr. Bacteriol., 1921, 5, p. 12.

Kendall,⁵ in advancing his theory of bromotherapy, takes the view that by maintaining a sufficient concentration of carbohydrate within the intestinal canal there should result a shifting of the metabolism of the intestinal organs, both pathogenic and nonpathogenic, so that harmless organic acids instead of protein decomposition products would be formed. Kendall and his co-workers have shown in vitro that such is the case, and that bacteria will get their energy from available carbohydrates in preference to proteins. He claims that under these conditions the intestinal contents become acid and consequently unfavorable for the development of proteolytic and pathogenic types.

TABLE 1
MEAT SERIES

Rat	Contents	Appearance	P _H	Gram Stain	Cultures
1, 3, 5, 7, 9, 11, 13, 15	Cecum	Dark color, disagreeable odor, indol present	7.0-7.1	95% slender or fusiform gram-negative bacilli and spirilla; an occasional gram-positive bacillus or diplococcus	Veillon: gas positive Plates: <i>B. acidophilus</i> 0.5%
17	Cecum, colon	Brown, foul odor	7.0-7.1	Approximately 80% gram-negative bacilli; remainder gram-positive bacilli and diplococci	Veillon: gas positive Plates: <i>B. acidophilus</i> 30%
19	Cecum	As 17.....	7.0-7.1	As 17.....	Veillon: gas in both cecum and colon tubes. Butyric acid odor Plates: approximately 25% <i>B. acidophilus</i> in both cases

17 and 19 were fed oats and meat, all the others were fed meat only.

Rettger and his collaborators, however, disagree with Kendall's explanation, and Rettger and Cheplin¹ have concluded that "the process of elimination of the ordinary mixed flora and the establishment of *B. acidophilus* apparently does not depend on any changes in the hydrogen-ion concentration within the intestine." They base their conclusions on determinations of the hydrogen-ion concentration of the feces of white rats and man with a simplified intestinal flora strongly dominated by *B. acidophilus*. They found that the actual acidity in such cases ran almost parallel with that observed with the usual mixed flora, and consequently they interpret the elimination of the proteolytic types as the result of "optimum cultural and environmental conditions" for the aciduric types, the slow absorption of lactose or dextrin allowing the sugars to reach the regions in which the bacterial change occurs.

⁵ Textbook of Bacteriology, 1921, p. 625.

TABLE 2
MEAT AND LACTOSE SERIES

Rat	Contents	Appearance	pH	Gram Stain	Cultures
2	Cecum	Yellowish-brown..	4.4	Practically 100% plump gram-positive, acidophilus-like rods; some large swollen gram-negative bacilli; apparently acidophilus-like rods, beaded	Veillon: no gas Plates: 90% acidophilus
4	Cecum	Brown, no offensive odor	4.4	As 2.....	As 2
6	Cecum	As 4, no indol present	4.4	As 2.....	As 2
8	Cecum	Semifluid, no offensive odor	4.6	As 2.....	Veillon: slight gas Plates: 90% acidophilus
	Colon	Well formed, no diarrhea			
10	Cecum	Fluid, no offensive odor	6.2	As 2.....	Veillon: no gas
	Colon	Semisolid, no diarrhea	6.4	As 2	
12	Cecum	Fluid, yellowish-brown	4.6	As 2.....	Veillon: no gas Plates: 99% acidophilus
	Colon	Semisolid, no diarrhea, no offensive odor			
14*	Cecum	Fluid, disagreeable odor	6.8	Plump gram-negative bacilli and gram-positive diplococci predominant; a few acidophilus-like rods	Veillon: gas positive Plates: 1% acidophilus
16	Cecum, colon	Semisolid, no offensive odor	4.8	As 2.....	Veillon: no gas; 95% acidophilus
18	Cecum	As 16.....	4.4	As 2.....	As 16
	Colon	No diarrhea			
20	Cecum	Semifluid, disagreeable odor	6.6	Approximately 75% gram-negative bacilli	Veillon: gas positive; 30% acidophilus
	Colon	Well formed.....	6.8	As cecum.....	As cecum
22	Cecum	Semisolid, disagreeable odor	6.4	50% plump gram-negative small bacilli	Veillon: gas positive
	Colon	Well formed, unpleasant odor	6.8	As cecum.....	As cecum: B. acidophilus approximately 35%
24	Cecum	Fluid.....	4.4	Predominance of acidophilus-like rods and large, plump, beaded, gram-negative bacilli in both cases	Veillon: no gas; 99% acidophilus in both cecum and colon
	Colon	Semifluid.....	5.6		
26	Cecum	As 24, no indol....	4.4	As 24.....	As 24
	Colon	Well formed, no offensive odor	5.8	As 24.....	As 24
28	Cecum	As 26.....	4.6	As 24.....	As 24
	Colon	As 26.....	6.0	As 24.....	As 24
30	Cecum	Semifluid, no indol	4.6	As 24.....	Veillon: no gas; 90% acidophilus
	Colon	Well formed.....	6.2	As 24.....	As cecum
32	Cecum	As 30.....	4.6	As 24.....	Veillon: slight gas; no butyric acid odor; 90% acidophilus
	Colon	As 30.....	5.0	As 24.....	As cecum
34	Cecum	Semisolid, no foul odor	4.4	As 24.....	As 24
	Colon	As 30.....	6.0	As 24.....	As 24
36	Cecum	As 34.....	4.4	As 24.....	Veillon: gas positive; 80% acidophilus
	Colon	As 30.....	5.6	As cecum.....	As cecum: no butyric acid odor

* Rat 14 refused to eat for 24 or more hours before being killed.

They base the latter idea on the demonstration of reducing substances in the feces of rats and man when fed sufficient quantities of lactose or dextrin.

The conclusions of Rettger and Cheplin necessarily assume the actual acidity of the contents of the higher regions of the large intestine to be the same as that of the feces themselves. As will be shown later, this is not the case. From a physiologic or pathologic standpoint, it is probable that the ill effects which may come from undue activity of intestinal bacteria are the results of bacterial action in the higher levels of the digestive tract where absorption is greater. In experimental intestinal obstruction, the toxemia developing is much more rapid in the upper than in the lower levels of the intestinal tract. Again, there is a steady decrease in the numbers of viable bacteria from the cecum down, until it is commonly stated that the feces are composed largely of dead bacterial cells. If bacterial action is responsible for toxemias of intestinal origin, the significant action must take place where the viable organisms are most numerous. Presumably this is in the cecum and upper colon, and in all probability the real transformation of the intestinal flora occurs here. If this reasoning is correct, we should determine the hydrogen-ion concentration of the intestinal contents in these regions and not that of the feces.

We find that there is a marked difference in the actual acidity of the contents of the cecum and lower colon when the determinations are made separately, the contents of the colon being much less acid than those of the cecum. This fact is analogous in some respects to the observations of McClendon, Shedlov and Karpman⁶ that in rabbits with long ileums the acidity decreased on the way down. McClendon suggested that this was probably due to the progressively greater absorption of carbon dioxide. If this is the case, anything which would hasten the passage of the contents along the tract would tend to prevent absorption or neutralization. This is probably the explanation of the effects of laxatives. Robinson⁷ has recently shown that in general the administration of laxatives results in a lowering of the P_H of the feces. He states that "diarrhea is accompanied by acidity and constipation by alkalinity of the excreta."

EXPERIMENTAL

The first step in the present study was to feed diets which would lead to intestinal floras that were either predominantly proteolytic or aciduric. Full-grown white rats were used, some being fed nothing but uncooked ground beef,

⁶ Jour. Biol. Chem., 1918, 34, p. 1.

⁷ Ibid., 1922, 52, p. 445.

and the others the same with the addition of a definite amount of lactose well mixed with the meat. One part of lactose to three parts of meat caused a pronounced simplification of the intestinal flora with *B. acidophilus* dominant, but with no resulting diarrhea. After a few days on these diets the rats were killed and the hydrogen-ion concentration of the contents of the cecum and lower colon determined, using Clark and Lubs indicators. At the same time Gram stains were made and cultural studies carried out on whey agar plates and in deep Veillon tubes of whey agar. The procedure in determining the actual acidity of the intestinal contents was as follows: 500 mg. of the material was suspended in 20 cc of practically neutral distilled water. This was centrifuged at high speed to eliminate turbidity, and the indicator then added. Brom-thymol-blue, brom-cresol-purple and methyl-red were the indicators used. The hydrogen-ion concentration was first determined roughly by adding a drop of the indicator to a few drops of the emulsion in a porcelain dish, and then the exact acidity was determined by diluting the emulsion with an equal volume of neutral water, adding the appropriate indicator and comparing in a comparator with LaMotte buffer mixtures ranging from P_H 4.4 to 7.2. The results of these determinations are tabulated in the accompanying protocol.

DISCUSSION

These experiments indicate that when a meat diet is fed to white rats the reaction of the contents of the cecum and colon is practically neutral; the contents are foul smelling and contain indol; and the flora is of a gas-producing, proteolytic type with *B. acidophilus* at a minimum. The addition of lactose to the meat in the proportions stated causes a distinct change in the bacterial picture and in the reaction of the contents of the cecum and colon. It appears significant to us that the simplification of the flora varies directly with the hydrogen-ion concentration. With few exceptions, when the P_H of the cecal contents was 4.4, the Gram stains showed a flora composed mainly of large acidophilus-like rods, and culturally a non-gas-producing flora in which *B. acidophilus* was present in great predominance. When the animals refused to eat after a time, as in the case of No. 14 and probably No. 20, the reaction was nearer neutrality, and at the same time *B. acidophilus* was not so prominent.

The striking feature, however, is the difference in the acidity of the cecal contents as compared with the contents of the lower colon of the same animal. This indicates in all probability that the acid is either absorbed or neutralized as the contents pass down the tract. Obviously, then, the determination of the hydrogen-ion concentration of the feces cannot tell us what is occurring higher up in the tract in the region of maximum bacterial activity, and most probably where the actual intestinal transformation takes place. This would indicate that the conclusions of Rettger and Cheplin are incorrect in so far as they

minimize the importance of the actual acidity in eliminating the ordinary mixed flora. Michaelis and Marcora,⁸ and Clark and Lubs⁹ have shown that with members of the colon group a P_H of about 5 is the limiting end-point of growth. The above experiments demonstrate an acidity well below this point, yet there were great numbers of viable aciduric bacteria. With these conditions of high acidity there was no diarrhea, and the contents of the lower colon were semisolid or well formed. Evidently then the diet was physiologic from the standpoint of peristalsis. In the white rat, at least, a P_H of 4.4 of the cecal contents may occur without a diarrhea resulting.

These observations cause us to believe that Kendall's interpretation of the factors involved in the transformation of the intestinal flora is more nearly correct, viz., that an available carbohydrate is acted on by both proteolytic and fermentative types of organisms, and as a result an acidity develops which is distinctly unfavorable for the former group. At the same time, the demonstration of Rettger and his co-workers that lactose and dextrin are slowly absorbed, suggests the probability that a more favorable environment results for the aciduric types. The effect in any case is a simplification of bacterial types with the elimination to a large extent of the usual colon groups.

SUMMARY AND CONCLUSIONS

The actual acidity of the intestinal contents of the cecum and colon of white rats is an important factor in the simplification of the intestinal flora. With a high animal protein diet the reaction of the contents of both the cecum and colon is P_H 7.0 to 7.1, whereas the addition of lactose in proper proportions may lead to a P_H in the cecum of 4.6 with a P_H of 6.2 in the lower colon. Therefore, determinations of the actual acidity of feces are of slight value in interpreting the reaction of the intestinal contents higher up.

The simplification of the intestinal flora varies directly with the hydrogen-ion concentration, a P_H of 7.0 being characteristic of a gas-producing proteolytic type, whereas an increasing acidity is characterized by a diminution of proteolytic types and their replacement by aciduric types mainly dominated by *B. acidophilus*.

⁸ Ztschr. f. Immunitätsf., O., 1912, 14, p. 170.

⁹ Jour. Biol. Chem., 1915, 22, p. 87.

PATHS OF INFECTION BY BACTERIUM ABORTUS IN RABBITS, GUINEA-PIGS AND MICE

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Since the discovery by Bang¹ of the organism now known as *Bacterium abortus*, many and different views regarding the channels of infection in infectious abortion of cattle have been held. During all these years, however, comparatively little of importance has been added to the knowledge of this bacterium, except, perhaps, in its relation to agglutination and complement fixation, studied in more recent years, and its occurrence in the milk of infected cows, and in the organs of artificially inoculated animals.

In his early investigations, Bang believed, as did others, that the chief avenues of infection were the vagina and alimentary tract. Bang, in addition, emphasized the possible importance of the male as a passive conveyor of infection. For many years the view that the disease is transmitted from cow to cow through the agency of the male has been quite generally held in this country. In more recent years, however, much doubt has been expressed from time to time as to the validity of such a view. Three things have contributed largely to the present open-mindedness as to the paths of infection in this disease. One of these is the observations of Schroeder and Cotton,² Evans,³ and others that *B. abortus* is eliminated in cow's milk; another, the repeated claims of different investigators, including Hadley and Lothe,⁴ and Rettger, White and Chapman,⁵ that attempts to inoculate heifers and cows, or to bring about infection of females by cohabitation with infected males, usually result in failure; and third, by the now well-known fact that not infrequently unbred heifers become infected during the period of sexual maturity.

EXPERIMENTS

In these experiments, attempts were made to infect guinea-pigs, mice and rabbits by mouth, and guinea-pigs and rabbits through the vagina and the urethra.

In the cultivation of *Bacterium abortus*, 1.5% agar medium containing Fairchild's peptone and adjusted to P_H 6.9 to 7.0 was employed. The inoculated tubes were sealed in quart fruit jars (Lightning) and incubated at 34 to 36 degrees, for from 4 to 6 days, the oxygen tension being diminished by the employment of 4 large slant agar tubes freshly

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¹ Ztschr. Thiermed., 1897, 1, p. 241.

² Bureau of Animal Industry, 1911.

³ Jour. Infect. Dis., 1918, 23, p. 354.

⁴ Jour. Am. Vet. Med. Assn., 1916, 50, p. 143.

⁵ Storrs Exper. Station Bull. 108, 1921.

streaked with *B. cereus*. The technic of the agglutination and complement-fixation tests was that adopted by Gibbs and Rettger.⁶

Nine different strains of *B. abortus* were used, namely: Bang, B and W, old strains from other laboratories; W 40, from the outside, and S 1, 2, 3, and 4, and H 2, all relatively recent isolations. Strains S 3 and 4 and H 2 in particular were quite new. In practically all experiments, 2 or more strains were employed, and, as a rule, a combination of one of the older with one or more of the newer.

FEEDING EXPERIMENTS WITH GUINEA-PIGS

First Series.—Two young females and one grown male were given oats soaked with suspensions of *B. abortus* (S 1 and 3, and W 40). The feeding was repeated with one or more of these strains after intervals of 3, 7 and 26 days.

On the 31st day after the first artificial feeding of *B. abortus*, the 3 pigs were bled from the superficial vein of the neck, and the serum tested by complement fixation and agglutination. All gave positive reactions by both methods. Six days after the bleeding, one of the pigs died. All organs appeared normal except the spleen, which was enlarged and covered with small raised grayish patches, more or less characteristic of *abortus* infection in guinea-pigs. Cultures of the spleen gave typical growth on agar of *B. abortus*, which when washed off with phenolized salt solution and tested against positive and negative cow serum reacted in dilutions of 1:50, 1:100 and 1:300, with positive serum, and failed to react with negative serum. The organism obtained from the spleen gave every other indication of being *B. abortus*. Five and one-half months after the first infection, one of the two remaining guinea-pigs showed indications of weakness and walked with more or less difficulty. It was killed, and the serum tested by agglutination and complement-fixation tests. Positive reactions were obtained. Cultures on the liver, spleen, uterus, kidney, bladder and heart failed, however, to give any evidence of existing *B. abortus* infection.

The third pig of this series was still alive at the end of 8 months.

Second Series.—The guinea-pigs were divided into 2 groups of 3 each, A and B. The 3 pigs of group A were given 2 gelatin capsules filled with a heavy salt suspension of *B. abortus* (W, S 1 and 4, and H 2) and made to chew and to swallow the contents and remnants of the capsules.

Each of the 3 guinea-pigs of group B received into the esophagus, through a small specially prepared capsule gun, 2 capsules like those used in group A.

On the day following, both groups of animals received the same treatment again.

Twenty days after the first feeding, one of the guinea-pigs in group B died. Eighteen days later a second pig of this group was found dead. Serum from one of these two pigs (the first) gave no agglutination, while that of the other was doubtful.

Four weeks after the first treatment, the 3 pigs in group A and the remaining guinea-pig in group B were given another capsule containing *B. abortus* suspension (Bang, B, S 1 and 2, and H 2).

Forty days later, or about 10 weeks after the first inoculation, the surviving pigs were killed and the serum tested by agglutination and complement fixation.

⁶ Jour. Immunol., 1920, 5, p. 399.

The reactions of all 4 pigs in both tests were positive. With perhaps one exception, the animals appeared to be in good health. With the exception of the spleen and the right testis of guinea-pig 1 in group A, the various organs appeared normal. The spleen was much enlarged, but no gray patches or lesions of any kind could be detected. In the same animal, the right testis was enlarged. Cultures from the enlarged spleen and the urinary bladder (guinea-pig 3, group A) gave *B. abortus*. Agglutination in dilutions at 1:300 was positive. Culture tests on the other organs of this pig and of the other 3 pigs were negative.

FEEDING EXPERIMENTS WITH RABBITS

Five small and one half-grown rabbit were fed on oats and corn which had been well soaked with a heavy suspension of *B. abortus* (Bang, B, S 1 and 4, and H 2). In addition, one rabbit was given 3 gelatin capsules filled with bacterial suspension. Capsules were given by means of the special glass tube used on some of the guinea-pigs.

In less than 3 days, 3 of the young rabbits died, possibly from eating excelsior in the bedding. After substituting other bedding, no more trouble was encountered. Cultures from different organs of the rabbits that died failed to show any growth, and agglutinations were negative. Seven days after the first attempted infection all survivors were given the same treatment again. Thirteen days later, they were bled from the ear vein, and the serum tested by agglutination and complement fixation.

All gave positive reactions with both tests. Twenty-four days after the first feeding, one of the small rabbits was found moribund. Postmortem, the lungs alone appeared abnormal and more or less pneumonic. Cultures of the organs for *B. abortus* gave negative results. Five days later, or one month after the first feeding, the half-grown rabbit was found dead. Here, again, the lungs were more or less congested and pneumonic, the spleen normal, and cultures for *B. abortus* negative.

FEEDING EXPERIMENTS WITH MICE

Fifteen full-grown mice were fed oats which had been liberally treated with a suspension of *B. abortus* (S 1, 3, 4, and W 40). One day later, and again on the 5th day, the mice were supplied with grain feed treated in the same way.

Sixteen days after the first feeding, 1 of the mice was killed; the agglutination test of the serum was negative. Cultures of the liver, spleen and kidneys gave no growth. Seven days later, the 14 remaining mice received the same treatment as before. Forty-six days after the beginning of the experiment, all were killed. Four gave positive agglutination tests in dilutions of 1:50 and 1:100, while the results of the other 10 were negative. Owing to the small amount of blood obtainable, no fixation tests were made.

The following attempts were made to transmit infection in mice through the mother to the offspring. A mouse with 5 sucklings was inoculated intraperitoneally with 0.5 cc of suspension of Bang, B, and S 4. Four days later the mouse was inoculated with 0.5 cc of S 4 in the same manner.

Five weeks after the first inoculation, all the mice were killed, and agglutination tests were positive in dilutions of 1:50 and 1:100. In the mother mouse, the spleen was much enlarged, and the surface was dotted with numerous white patches which were not raised. The other organs were normal. Cultures from the kidney gave pure growth of *B. abortus*. None of the organs of the small mice showed any evidence of *B. abortus* infection. Cultures of the testes of the 4 males gave negative results.

The positive cultures from the kidney of the large mouse showed that the mouse was infected with *B. abortus*, and the agglutination by the serum was the result of the infection. Whether or not the agglutinations obtained with each of the small mice were due to actual infection of the mice through the milk of the mother, or to transmission of agglutinins from the mother to the young, must remain an open question. However, since the organs of the small mice appeared to be normal, there is the probability that the agglutinins had been transmitted. Furthermore, it is generally believed that, except in rare instances, infection is not transmitted directly from the mother to the young.

INFECTION BY WAY OF THE REPRODUCTIVE SYSTEM AND THE URETHRA

Guinea-Pigs.—In the first experiments of this series, the prepuce of 2 male guinea-pigs was drawn back and the glans penis painted with a heavy suspension of strains S 1 and 3, and W 40. An adult female was placed in the cage with one of these males. Fifty days later, the animals were bled and the serum tested as to agglutination and complement fixation, but with negative results.

In the second experiment, a suspension of *B. abortus* (S 1 and 3, and W 40) was painted on the inner surface of the urethra of a male pig. A grown female was placed in the same cage. Thirty days later the blood of the male gave positive agglutination and complement fixation reactions, while that of the female was negative. Forty-eight days later, the male pig was found dead, after having appeared ill for about 2 weeks. The spleen was enlarged and the surface covered with grayish spots. Both testes were enlarged and more or less soft.

Cultures were made from the liver, spleen and testes. From one of the testes alone, growth was obtained on agar medium which proved to be *B. abortus*. It was agglutinated by positive cow serum in dilutions of 1:50 to 1:300.

In exper. 3, the inner surface of the vagina of a female pig was painted with a suspension of strains S 1 and 3, and W 40. Forty-eight days later, the serum of this animal gave positive agglutination and complement fixation.

In exper. 4, the inner surface of the vagina of each of 2 female pigs was painted with a suspension of strains S 1 and 3, and W 40. A male pig was placed in the cage with the 2 females.

Thirty days after the vaginal infection, the 2 females gave indications of ill health. Agglutination and complement-fixation tests at this time gave positive reactions for both females and negative for the male.

Forty-six days later, one of the females died, and the other succumbed after an additional period of 50 days. *Bacterium abortus* could not be recovered from any of the organs.

In exper. 5, a male guinea-pig was placed in the cage with a female which had been artificially infected through the vagina, and which had quite recently given a positive agglutination and complement-fixation reaction with *B. abortus*. Thirty days later the blood serum of the male gave negative blood reactions.

Rabbits.—A heavy suspension of *B. abortus*, strains Bang, B, S 1 and 4, and H 2, was painted on the inner surface of the urethra of an adult male rabbit. On the following day, an adult female rabbit was placed in the same cage.

Twenty and twenty-nine days later, agglutination and complement fixation tests were positive in the male and negative in the female.

In a second experiment, the inner surface of the vagina of an adult female was painted with a heavy suspension of the strain just mentioned. Twenty days later, 3 small rabbits were born, which died within 5 days. The mother,

at this time, failed to react to agglutination, and gave doubtful fixation. However, as the serum of this rabbit was anticomplementary, little significance can be attached to the result.

Forty-eight days after the infection, the same female was treated as before with bacterial strain W, and 10 days later the agglutination test was still negative.

SUMMARY

The results show that guinea-pigs, rabbits and mice are highly susceptible to infection by *B. abortus*. Infection of these animals was easily brought about through the mouth, vagina and urethra. Of the three species, the mouse appears the least susceptible.

These observations form an interesting contrast to those carried out in recent years on cattle, particularly by Rettger and White, who through a long series of experiments demonstrated that artificial infection of the bovine species of animal by mouth is extremely difficult. Rettger and White⁵ failed to bring about positive agglutination and complement fixation or any other indications of infection, including abortion, in calves and maturing heifers which were daily fed large amounts of *B. abortus* suspensions prepared from the cultures of numerous strains, both old and new. They were able, however, to convert nonreacters into complete, and in a number of instances, permanent reactors and aborters, by vulvar application and intra-urethral injection of suspensions of *Bacterium abortus*. Such changes were brought about, not only in heifers and young cows which had not been employed in previous experiments, but also in a number of animals which had been subjected daily and for long periods of time to oral infection, or to intravaginal injections of suspensions of *B. abortus*.

A survey of the investigations conducted on cattle and on smaller animals leads to the conclusion that guinea-pigs, rabbits and mice constitute a group of extremely susceptible animals, while the bovine species is relatively resistant, especially to artificial infection.

It must be admitted that positive serologic reactions are not in themselves absolute proof of existing infection. There can be no doubt, however, that such reactions, except in very young, nursing animals, indicate either present or recent infection, and that they are therefore of great value in the study of transmission in cattle and other animals.

The probable transmission of agglutinins and other antibodies from mother to offspring is of interest, as is also the finding of *B. abortus* in the urinary bladder of one of the infected guinea-pigs.

The negative results obtained by the mating of previously unexposed with artificially infected and reacting animals are perhaps surprising.

However, the experiments were few, and actual mating was not observed. Furthermore, attempts to bring about infection in cattle through the vagina have often proved unsuccessful.

In experiments of the kind reported in this paper, there is always a possibility of infection having taken place through other routes than those which are singled out for trial. For example, oral administration cannot be conducted in such a way as to preclude infection through an abrasion of the mouth or esophagus, or by direct or indirect infection of the vulva and urethra. In larger animals, as for example the bovine species, the problem can be simplified, at least partly.

STRUCTURE OF COMPLEMENT

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The sap of the plant *Cotyledon scheideckeri*, the properties of which formed the subject of previous studies,¹ possesses also the faculty of inactivating complement (table 1).

The experiments were carried out as follows: The effective dose of complement (diluted 1:10) was added to 0.5 cc of the dilution of cotyledon sap indicated in the table; the mixture was shaken and immediately after this were added the hemolytic serum (0.5 cc of the triple dissolving dose) and 0.5 cc of a 5% suspension of red corpuscles of sheep. Into the tube was then poured isotonic salt solution until the quantity of liquid was 2.5 cc; this was kept at 37 C. for 1 hour.

The experiments in table 2 were undertaken to ascertain the relation between the precipitation in the serum due to the cotyledon sap and the inactivation of the complement.

The degree of precipitation was estimated immediately after the addition of the complement to the sap, and was defined as "strong," "slight," or "no precipitation." Thus the precipitation in the serum is not always followed by the inactivation of the complement. The experiments in table 3 likewise confirm this conclusion.

Applying the method described, we often established in our experiments the inactivation of the complement, though in fact there was no real inactivation, that is destruction of the complement or its chemical decomposition, in these instances. This is clearly shown by the following procedure: after decanting the liquid from the sediment by means of centrifugation, the complement is found in the liquid; without this intervention, no complement is discovered in the mixture including the precipitate (table 3).

The experiment was made as follows: Three cc of complement (effective dose 0.03) in a 1:10 dilution were mixed with 0.5 cc of cotyledon sap (not diluted). Three tubes were tested in regard to the contents of complement: (1) 0.5 cc of the mixture—tube 1; (2) 0.5 cc of the liquid decanted from the precipitate by means of centrifugation—tube 2; (3) 0.5 cc of the suspension of precipitate washed in isotonic salt solution (0.5 cc of the mixture was centrifuged and the remaining precipitate washed three times in isotonic salt solution)—tube 3.

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¹ Kritchevsky, I. L.: *Ztschr. f. Immunitätsforsch.*, O., 1914, 22, p. 381; 23, p. 331; *J. Exper. Med.*, 1917, 26, p. 669.

TABLE 1
EFFECT OF SAP OF COTYLEDON ON COMPLEMENT *

Plant No.	Degree of Dilution of the Sap of Cotyledon					Standard of Complement
	1 : 3	1 : 7	1 : 10	1 : 20	1 : 30	
1	—	—	N.	N.	—	0.03
2	—	—	N.	C.	C.	0.03
3	—	—	N.	C.	C.	0.03
4	—	—	I.	C.	C.	0.03
5	—	—	N.	N.	I.	0.03
6	—	—	N.	N.	ALC.	0.07
7	N.	N.	—	—	—	0.03
8	N.	N.	—	—	—	0.03
9	N.	N.	—	—	—	0.03
10	—	—	N.	C.	C.	0.03
11	I.	—	—	—	—	0.03
12	N.	N.	—	—	—	0.03
13	N.	N.	—	—	—	0.03
14	N.	C.	—	—	—	0.03
15	N.	N.	—	—	—	0.03
16	N.	N.	—	—	—	0.03
17	—	—	N.	Sl.	C.	0.03
18	—	—	N.	C.	C.	0.03
19	—	—	N.	C.	C.	0.03
20	—	—	N.	I.	—	0.03

In the tables, C. indicates complete hemolysis; ALC., almost complete hemolysis; Sl., slight hemolysis; N., no hemolysis; I., incomplete hemolysis.

TABLE 2
RELATION BETWEEN PRECIPITATION IN SERUM DUE TO COTYLEDON SAP AND INACTIVATION OF COMPLEMENT

Plant No.	Precipitation			Inactivation		
	Degree of Dilution of Sap			Degree of Dilution of Sap		
	1 : 10	1 : 20	1 : 30	1 : 10	1 : 20	1 : 30
2	Strong	Strong	Faint	N.	C.	C.
3	Strong	Faint	None	N.	C.	C.
4	Faint	None	None	I.	C.	C.
5	Strong	Faint	Faint	N.	N.	I.
6	Strong	Strong	Faint	N.	N.	ALC.
10	Faint	None	None	N.	C.	C.
18	Strong	Faint	Faint	N.	C.	C.

TABLE 3
RESULTS OF TESTS IN REGARD TO CONTENT OF COMPLEMENT

Plant No.	Tube No.		
	1	2	3
2.....	N.	I.	N.
3.....	N.	C.	N.
6.....	N.	N.	N.
7.....	N.	C.	N.
8.....	N.	C.	N.
9.....	N.	C.	N.
10.....	N.	C.	N.
13.....	N.	C.	N.
16.....	N.	C.	N.
17.....	N.	C.	N.
19.....	N.	C.	N.

We believe that these facts are most plausibly explained if we admit that we are dealing, not with the destruction of complement or with its chemical decomposition under the action of the cotyledon sap, but with the absorption of the complement by the precipitate. It is to be noticed, however, that the precipitate does not always absorb the complement; precise conditions are required for it. It is probable that the presence of hemolytic serum is one of these conditions, the sediment and hemolytic amboceptor acquiring a greater avidity in regard to the complement than the sediment alone, formed by the addition of cotyledon sap to the serum of a guinea-pig. Thus one may interpret the

TABLE 4
RESULTS OF EXPERIMENTS WITH HEATED AND UNHEATED SERUM

Plant No.	I. Experiments with Heated Serum (One-Half Hour 54 F.)				II. Experiments without Heated Serum			
	1 : 3	1 : 7	1 : 10	1 : 20	1 : 3	1 : 7	1 : 10	1 : 20
1	C.	C.	N.	N.
2	C.	N.	..
3	C.	N.	..
4	C.	I.	..
5	Sl.	C.	N.	N.
6	N.	C.	N.	N.
7	N.	C.	N.	N.
8	Sl.	C.	N.	N.
9	N.	C.	N.	N.
10	A.C.	N.	..
12	C.	C.	N.	N.
13	N.	N.	N.	N.
14	C.	N.
15	N.	N.	N.	N.
16	N.	N.
17	N.	C.	N.	Sl.
18	C.	N.	..
19	C.	N.	..

cases when no complement was discovered in the presence of the sediment, and yet was found in a free state in the decanted liquid.

On the other hand, the addition of guinea-pig serum kept at 54 F. for one-half hour makes the absorption of the complement by the precipitate impossible in the majority of cases (table 4).

Of course it is possible to explain this effect of the serum kept at 54 F. for one-half hour according to the theory of Ritz, who attributed it to the reconstruction of the complement (which had lost its third component under the action of the sap) through the third component present in the heated serum added afterward.

The experiments were made as follows: Five-tenths c.c of the dilution of cotyledon sap indicated in the table was added to the effective dose of com-

plement (table 1) and mixed with the same quantity of guinea-pig serum kept at 54 F. for one-half hour; then the hemolytic system was added, and the tube was left at 37 F. for 1 hour (tube 1). The control experiments were made with the same sap without the hot serum (tube 2).

If one considers, however, the inactivation as a result of the absorption of the complement, one must assume that the albugineous substances of the hot serum acquire defensive functions in regard to the complement.

Guinea-pig serum retains its protecting functions at 65 F. and even at 75 F. for 1 hour, while the third component of the complement is unable to withstand a higher temperature than 54 F., according to Ritz (table 5).

TABLE 5
RESULTS OF EXPERIMENTS WITH SERUMS AT VARIOUS TEMPERATURES

Plant No.	65 F.		75 F.		Experiment without Heated Serum	
	1 : 10	1 : 20	1 : 10	1 : 20	1 : 10	1 : 20
2	C.	..	C.	..	N.	..
3	C.	..	C.	..	N.	..
5	Sl.	C.	..	C.	N.	N.
6	N.	C.	N.	C.	N.	N.

TABLE 6
EFFECTS OF VARIOUS QUANTITIES OF PROTECTING COLLOIDS

Plant No	Quantity of Heated Serum (One-Half Hour 54 F.)							
	0.3	0.2	0.1	0.08	0.05	0.01	0.005	0.001
3	C.	C.	Al.C.	Al.C.	N.	N.	N.	N.
6	C.	Al.C.	Al.C.	Al.C.	N.	N.	N.	N.

The quantity of protecting colloids, in the form of heated guinea-pig serum, must not be less than a certain limit, or they are unable to prevent the absorption of the complement (table 6).

SUMMARY

The sap of *Cotyledon scheideckeri* has the power of inactivating guinea-pig complement.

The precipitation in the serum is not always followed by the inactivation of complement.

We are dealing, not with the destruction of the complement or with its chemical decomposition under the action of the cotyledon sap, but with the absorption of the complement by the precipitate.

The precipitate does not always absorb the complement, but precise conditions are required for it; the presence of hemolytic serum is one of these conditions.

The presence of guinea-pig serum heated at 54 F. for one-half hour makes the absorption of the complement by the precipitate impossible in the majority of cases.

Guinea-pig serum conserves its protecting functions against complement heated at 65 F. and even at 75 F. for 1 hour.

THE RELATION OF IMMUNITY REACTIONS TO THE BIOGENETIC LAW

INVESTIGATIONS OF THE CHEMICAL STRUCTURE OF THE PROTOPLASM OF ANIMALS DURING EMBRYONIC DEVELOPMENT BY MEANS OF HETEROGENEOUS HEMOLYSINS

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I have already shown¹ that the biochemical properties of protoplasm are subject to transformation during the embryonic period. The reaction of the deviation of complement served to prove that the chemical structure of the protoplasm of a frog embryo (9th week) differed from that of the adult form (imago).

The experiments now described were undertaken to determine the same problem in regard to the hen (*Gallus domesticus*). Doerr and Pick discovered in the organs of the hen and Kritchevsky in the red corpuscles of the hen the presence of a heterogeneous antigen producing lysins against the red corpuscles of the sheep. On this account I wished to ascertain whether the chemical properties of the chick embryo were identical with those of the adult hen; in other words, whether the cells of the embryo possess the heterogeneous antigen found in the organs of the hen, and if they do, at what stage of their development this antigen appears. For this purpose rabbits were immunized with hen's eggs and with embryos of different ages, as described in the protocols.

The hemolytic experiment was conducted as follows: three c c of liquid were placed in each tube; the quantity of complement used was 0.1 c c which alone is unable to dissolve red corpuscles; the quantity of red corpuscles of sheep used was 1 c c of a 5% suspension; the tubes were kept at 37 C. for 1 hour.

IMMUNIZATION OF RABBITS WITH THE WHITE AND YOLK OF HEN'S EGG

Before the injection the white and yolk were mixed with equal parts of isotonic salt solution.

Rabbit 1.—Weight 1,480 gm.; Oct. 16; received 2.5 c c of the white and yolk of an egg into the ear vein. Oct. 23, 0.25 c c into the vein and 3 c c into the abdomen. Oct. 31, hemolytic experiment: 0.05 c c, incomplete hemolysis; 0.025 c c and 0.01 c c, no hemolysis.

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¹ *Centralbl. f. Bakteriol.*, I, O., 1914, 72, p. 81.

Rabbit 2.—Weight 1,400 gm.; Nov. 1; received 5 cc of white and yolk in the ear vein. Nov. 11, 6 cc into the ear vein. Nov. 19, hemolytic experiment: 0.05 cc, almost complete hemolysis; 0.025 cc and 0.01 cc, no hemolysis.

Rabbit 3.—Weight 1,260 gm. Nov. 11, received 3.5 cc of white and yolk into the ear vein. Nov. 24, 5 cc into the abdomen. Dec. 1, hemolytic experiment: 0.05 cc, complete hemolysis, 0.025 cc, almost complete hemolysis; 0.01 cc, slight hemolysis; 0.005 cc, no hemolysis.

According to Forssman,² this amount of hemolysins is found even in normal rabbits and does not depend on immunization; according to Doerr and Pick³ 0.0008 cc of serum is able to produce complete hemolysis of red corpuscles of sheep in nonimmunized rabbits. Therefore only those hemolysins are immune which are found in quantities of serum smaller than the dose indicated.

These experiments show that the hen's egg does not contain heterogeneous antigen. The discovery of heterogeneous antigen in the embryo would have raised the objection that the antigen is not contained in the protoplasm of the embryo and might be derived from the second membranes or the nutritive yolk.

In the following experiments the rabbits were immunized with the yolk of the hen's egg, containing formative yolk (the spot).

Rabbit 4.—Weight 1,950 gm.; Nov. 11; received the formative yolk of 6 eggs. Nov. 22, received the formative yolk of 8 eggs into the ear vein. Dec. 1, hemolytic experiment: 0.05 cc, almost complete hemolysis; 0.025 cc, incomplete hemolysis; 0.0125 cc, slight hemolysis; 0.01 cc, no hemolysis.

Rabbit 5.—Weight 2,000 gm.; Dec. 7, received the formative yolk of 8 eggs into the ear vein. Dec. 6, received the formative yolk of 8 eggs into the abdomen. Dec. 22, hemolytic experiment: 0.05 cc, complete hemolysis; 0.025 cc, incomplete hemolysis; 0.0125 cc and 0.01 cc, traces of hemolysis; 0.006 cc, no hemolysis.

Rabbit 6.—Weight 2,145 gm.; Dec. 7, received the formative yolk of 6 eggs into the ear vein. Dec. 16, received the formative yolk of 6 eggs into the abdomen. Dec. 22, hemolytic experiment: 0.05 cc and 0.025 cc, complete hemolysis; 0.0125 cc, incomplete hemolysis; 0.01 cc, incomplete hemolysis; 0.006 cc and 0.005 cc, no hemolysis.

Thus the rabbits immunized with the formative yolk do not produce heterogeneous hemolysins.

In the experiments now described the rabbits were immunized with hen embryos of different ages: 2, 4, 6, and 11 days. The embryos were taken from the egg, washed three times in isotonic salt solution and weighed, then thoroughly rubbed in an isotonic salt solution, and the suspension injected into rabbits.

IMMUNIZATION OF RABBITS WITH 2 DAY EMBRYOS

Rabbit 7.—Weight 1,020 gm.; April 5, 4 embryos injected into the ear vein. April 14, 4 embryos injected into the ear vein and the abdomen (0.25 cc into

² Biochem. Zeitschr., 1910, 23, p. 146.

³ Zeitschr. f. Immunitätsf., 1913, 19, p. 251.

the ear vein, 4 c c, into the abdomen). Test on April 24. Hemolytic experiment: 0.025 and 0.012 c c, complete hemolysis; 0.01 c c, almost complete hemolysis; 0.005 c c, slight hemolysis; 0.003 c c, no hemolysis.

Rabbit 8.—Weight, 1,175 gm.; April 5, 4 embryos injected into the ear vein. April 14, 5 embryos into the ear vein and the abdomen (0.25 c c into the vein and 5.25 c c into the abdomen). Tests on April 21 and 24; both times the results of the hemolytic experiment were the same: 0.025, 0.012 and 0.01 c c, no hemolysis.

IMMUNIZATION OF RABBITS WITH 4 DAY EMBRYOS

Rabbit 9.—Weight 855 gm.; April 3, 0.12 gm. of embryos (2.2 embryos). April 11, 0.11 gm. of embryos (2.5 embryos) injected into the ear vein. Test on Dec. 19, hemolytic experiment: 0.025, 0.0125, 0.01, and 0.005 c c, complete hemolysis; 0.003 c c, incomplete hemolysis.

Rabbit 10.—Weight, 975 gm.; April 3, 0.14 gm. of embryos (2.6 embryos). April 11, 0.11 gm. of embryos (2.5 embryos) injected into the ear vein. Test on April 19, hemolytic experiment: 0.025, 0.012, 0.01, 0.005, and 0.003 c c, complete hemolysis; 0.002 c c, almost complete hemolysis.

IMMUNIZATION OF RABBITS WITH 6 DAY EMBRYOS

Rabbit 11.—Weight 1,000 gm.; Feb. 26, 0.5 gm. of embryos. March 5, 1.06 gm. of embryos injected into the ear vein. Test on March 12, hemolytic experiment: 0.01, 0.005, and 0.003 c c, complete hemolysis; 0.0025 and 0.002 c c, almost complete hemolysis.

IMMUNIZATION OF RABBITS WITH 11 DAY EMBRYOS

Rabbit 195.—Weight 1,400 gm.; Feb. 24, 1.56 gm. of embryos injected into the ear vein. March 2, 1.65 gm. of embryos into the ear vein and the abdomen (equal parts). Test on March 10, hemolytic experiment: 0.01, 0.005, 0.003, 0.002, and 0.00125 c c, complete hemolysis; 0.001 c c, almost complete hemolysis.

The experiments show that the heterogeneous antigen producing hemolysins against red corpuscles of sheep appears only in relatively advanced periods of the embryonic development of the hen; that is, not before 4 days after the division of the egg began. In earlier phases (2 days) the protoplasm of the embryo does not possess heterogeneous sheep antigen.

The facts presented in this article suggest the conclusion that the biochemical properties of animal cells are subject to transformations during the ontogenetic development (absence of heterogeneous sheep antigen in the egg and the 2 day embryos, its presence in 4 day embryos, and in later stages of development).

As regards the hen, it should be stated that the chemical structure of the cellular nucleus also changes during the ontogenesis. My earlier investigations⁴ have proved that the heterogeneous sheep antigen, producing lysins against red corpuscles of sheep, is contained in the cellular nucleus.

⁴ Jour. Exper. Med., 1916, 24, p. 233.

SUMMARY

The hen's egg and the formative yolk do not contain heterogeneous sheep antigen.

In earlier phases of the development (2 days), the protoplasm of the hen embryo does not possess heterogeneous sheep antigen.

The heterogeneous sheep antigen producing hemolysins against red corpuscles of sheep appears only in relatively advanced periods of the development of the hen; that is, not before 4 days after the division of the egg began. Therefore we can state that the biochemical properties of animal cells are subject to transformation during the ontogenetic development.

HETEROGENEOUS ANAPHYLAXIS

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Forssman and others have shown that lysins against red blood corpuscles of sheep may be obtained through immunization with the organs of animals which are far removed from the sheep phylogenetically or with the nucleus of the red corpuscles of certain species of animals (Kritchevsky). This refutes the theory of the specificity of antibodies. The experiments described in the present study prove that rabbits immunized with the cells of some animal species furnish, not only lysins against the red corpuscles of a foreign species—heterogeneous hemolysins, as they are called by Friedberger—but also other heterogeneous antibodies. One may thus speak of heterogeneous antibodies in a general way. Some time ago I¹ showed that rabbits immunized with red corpuscles of the hen produce a lysin against red corpuscles of sheep. These facts made me believe that rabbits immunized with red corpuscles of the hen should produce not only hemolysins but also heterogeneous anaphylactic antibodies, as occurs with homologous antibodies. This hypothesis was confirmed by the experiments described in the protocols and table 1.

In the majority of cases, the rabbits were sensitized twice with red blood corpuscles of the hen freed from serum by washing. After a fixed time the anaphylactic power was tested by means of red corpuscles of sheep injected into the ear vein. On the same day I determined the quantity of lysins against red corpuscles of sheep. Twelve of the twenty-three animals sensitized with red corpuscles of the hen died of anaphylaxis or showed the picture of anaphylactic shock characteristic for rabbits after the intravenous injection of red corpuscles of sheep. An intense anaphylactic shock was observed in six instances. The clinical symptoms of heterogeneous anaphylaxis are not described in the protocols, because they were the same in all the animals, with slight individual variations.

The intense anaphylactic shock usually begins with convulsions of the body and extremities, but sometimes the convulsions are preceded

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¹ Jour. Exper. Med., 1916, 24, p. 233.

by a brief period of restlessness, revealed by manege movements or by the inability to move and run. The intervals between the clonic convulsions become longer each time; during these intervals the animal suffers dyspnea, and the eyes roll. The reflected excitability increases: each touch causes new convulsions; the temperature falls progressively; the reflexes of the cornea disappear and the animal dies; opisthotonos is often irreparable, the head of the dead animal sometimes being thrown back at right angles to the body. At necropsy the heart is still beating; now and then thrombi are found in its cavities. The lungs are usually shrunk; in one case only they were greatly inflated, and filled the thorax, partly covered the heart, and were edematous.

PROTOCOLS

Rabbit 1.—Weight, 1,520 gm.; Oct. 27, 1.5 cc² of red corpuscles of the hen were injected into the vein; Nov. 3, 0.25 cc + 1.25 cc³ injected into the vein. Nov. 10, hemolytic experiment: 0.002 cc, complete hemolysis of red corpuscles of sheep; 0.0014 cc, incomplete hemolysis; 0.0011 cc, no hemolysis. Nov. 11, 4 cc of sheep corpuscles injected. Death after 11 hours. Thrombi in the right auricle.

Rabbit 2.—Weight, 1,345 gm.; Oct. 27, 1.5 cc of red corpuscles of the hen were injected into the vein; Nov. 3, 0.5 cc + 1.25 cc injected. Nov. 10, hemolytic experiment: 0.0011 cc, complete hemolysis of red corpuscles of sheep; 0.001 cc, almost complete hemolysis; 0.0008 cc, no hemolysis. Nov. 11, 1 cc of red corpuscles of sheep were injected into the vein. Immediately after the injection (after 25 seconds) began the anaphylactic shock. Death 2 minutes after the beginning of the experiment. Thrombus in the right auricle.

Rabbit 3.—Weight, 1,040 gm.; Nov. 8, 1.25 cc of red corpuscles of the hen were injected into the vein. Nov. 19, 2 cc into the abdomen. Nov. 27, 0.25 cc into the vein and 1.5 cc into the abdomen. Dec. 4, hemolytic experiment: 0.003 cc, complete hemolysis of red corpuscles of sheep; 0.002 cc, incomplete hemolysis; 0.001 cc, incomplete hemolysis; 0.0008 cc, slight hemolysis; 0.0006 cc, no hemolysis. Dec. 4, 1.5 cc of red corpuscles of sheep injected into the vein. After the injection paresis of the extremities and extreme weakness appeared. Six minutes after the beginning of the experiment the anaphylactic shock occurred, and one minute later the animal died. The lungs were edematous and filled the entire thorax and covered the heart. Thrombi in the right auricle.

Rabbit 4.—Weight, 1,125 gm.; Nov. 9, 1 cc of red corpuscles of the hen were injected into the vein. Nov. 19, 1.25 cc into the abdomen. Nov. 26, hemolytic experiment: 0.002 cc, complete hemolysis of red corpuscles of sheep; 0.0016 cc, almost complete hemolysis; 0.0014 cc, incomplete hemolysis; 0.0012 cc, no hemolysis. Nov. 26, 3 cc of red corpuscles of sheep were injected into the vein. After the injection no pathologic phenomena were observed at first; then gradually appeared paresis of the extremities, extreme weakness, and a comatose state in which the animal died 2 hours after the beginning of the experiment. No thrombi were found in the heart.

² The quantities indicate the number of cubic centimeters of the sediment of red corpuscles, washed three times in isotonic salt solution.

³ At the second sensitization the quantities indicated were injected separately, at intervals of one-half to one hour, in order to avoid homologous anaphylaxis.

Rabbit 5.—Weight, 1,140 gm.; Nov. 8, 1.25 cc of red corpuscles of the hen were injected into the vein. Nov. 19, 2 cc into the abdomen. Nov. 26, hemolytic experiment: 0.01 cc, complete hemolysis; 0.003 cc, slight hemolysis; 0.0025 cc, no hemolysis; 1.75 cc of red corpuscles of sheep were injected into the vein with no pathologic effect.

Rabbit 6.—Weight, 1,380 gm.; Nov. 9, 1 cc of red corpuscles of the hen were injected into the vein; Nov. 20, 2.5 cc into the abdomen. Nov. 26, hemolytic experiment: 0.0016 cc, complete hemolysis; 0.0012 cc, incomplete hemolysis; 0.0011 cc, slight hemolysis; 0.001 cc, no hemolysis. Nov. 26, 2.5 cc of red corpuscles of sheep injected into the vein. Death after 10 hours. Thrombus in the right side of the heart.

Rabbit 7.—Weight, 880 gm.; Nov. 9, 1 cc of red corpuscles of the hen. Nov. 19, 1.5 cc injected into the vein. Nov. 26, hemolytic experiment: 0.0011 cc, complete hemolysis; 0.001 cc, almost complete hemolysis; 0.0008 cc, no hemolysis. Nov. 26, 3 cc of red corpuscles of sheep injected intravenously. After the injection paresis of extremities and extreme weakness passing into a comatose state. Death after 4 hours. Thrombi in the right side of the heart.

Rabbit 8.—Weight, 1,070 gm.; Nov. 19, 1.5 cc of red corpuscles of the hen were injected into the vein; Nov. 27, 0.25 cc + 1.5 cc injected. Dec. 4, hemolytic experiment: 0.01 cc, complete hemolysis; 0.003 cc slight hemolysis; 0.0025 cc, no hemolysis. Dec. 4, 1.5 cc of red corpuscles of sheep injected into the vein. No pathologic phenomena were observed.

Rabbit 9.—Weight, 1,000 gm.; Nov. 9, 1 cc of red corpuscles of the hen; Nov. 19, 1.5 cc, and Nov. 27, 2.5 cc injected intravenously. Dec. 4, hemolytic experiment: 0.01 cc, complete hemolysis; 0.005 cc, slight hemolysis; 0.003 cc, no hemolysis. Dec. 4, 2 cc of red corpuscles of sheep injected into the vein. Death after 6 hours. No thrombi in the heart.

Rabbit 10.—Weight, 810 gm.; Nov. 9, 1 cc of red corpuscles of the hen were injected into the vein; Nov. 20, 2.5 cc injected into the abdomen. Nov. 26, hemolytic experiment: 0.0016 cc, complete hemolysis; 0.0014 cc, incomplete hemolysis; 0.0012 cc, slight hemolysis; 0.0011 cc, no hemolysis. Nov. 26, 2.5 cc of red corpuscles of sheep injected into the vein. Death after 5 hours. Thrombi in the right side of the heart.

Rabbit 11.—Weight, 1,060 gm.; Nov. 20, 1.25 cc of red corpuscles of the hen, and Nov. 27, 0.25 cc + 1.5 cc injected into the vein. Dec. 12, hemolytic experiment: 0.005 cc, almost complete hemolysis; 0.003 cc, incomplete hemolysis; 0.002 cc, slight hemolysis; 0.0016 cc, no hemolysis. Dec. 12, 2.5 cc of red corpuscles of sheep injected into the vein with no pathologic effect.

Rabbit 12.—Weight, 1,070 gm.; Nov. 20, 1.25 cc, and Nov. 27, 1.75 cc of red corpuscles of the hen were injected intravenously. Dec. 5, hemolytic experiment: 0.003 cc, complete hemolysis; 0.0025 cc, almost complete hemolysis; 0.002 cc, almost complete hemolysis; 0.0012 cc, incomplete hemolysis; 0.001 cc, slight hemolysis; 0.0008 cc, no hemolysis. Dec. 5, 1.5 cc of red corpuscles of sheep injected into the vein. After 3 minutes anaphylactic shock began, and 2 minutes later the animal died. Thrombi in the right auricle and ventricle.

Rabbit 13.—Weight, 1,800 gm.; Nov. 28, 2 cc, and on Dec. 12, 0.25 cc + 1.25 cc of red corpuscles of the hen were injected into the vein. Dec. 12, hemolytic experiment: 0.01 cc, complete hemolysis; 0.005 cc, almost complete hemolysis; 0.0025 cc, incomplete hemolysis; 0.002 cc, slight hemolysis; 0.0016 cc, no hemolysis. Dec. 12, 2 cc of red corpuscles of sheep injected intravenously. After one-half minute paresis of the extremities appeared; then a strong dyspnea began, followed by the typical anaphylactic shock; after 2 min-

utes the convulsions ceased, the animal returned to its usual sitting position, but still remained in a state of apathy and extreme weakness, then recovered gradually and became normal again.

Rabbit 14.—Weight, 1,470 gm.; Dec. 5, 1.5 cc of red corpuscles of the hen, and Dec. 13, 0.25 cc + 1.5 cc injected into the vein. Dec. 22, hemolytic experiment: 0.01 cc, complete hemolysis; 0.005 cc, incomplete hemolysis; 0.003 cc, slight hemolysis; 0.002 cc, no hemolysis. Dec. 22, 1.5 cc of red corpuscles of sheep injected into the vein. No pathologic phenomena were observed.

Rabbit 15.—Weight, 1,870 gm.; Dec. 5, 1.5 cc, and Dec. 13, 0.25 cc + 1.5 cc of red corpuscles of the hen were injected intravenously. Dec. 22, hemolytic experiment: 0.01 cc, complete hemolysis; 0.005 cc, slight hemolysis; 0.003 cc, no hemolysis. Dec. 22, 2 cc of red corpuscles of sheep injected into the vein with no pathologic effect.

Rabbit 16.—Weight, 1,680 gm.; Jan. 19, 1.5 cc, Jan. 25, 0.5 cc + 1 cc, and Feb. 4, 0.5 cc + 1 cc of red corpuscles of the hen were injected intravenously. Feb. 12, hemolytic experiment: 0.01 cc, incomplete hemolysis; 0.005 cc, slight hemolysis; 0.003 cc, no hemolysis. Jan. 12, 2 cc of red corpuscles of sheep injected into the vein. No pathologic phenomena.

Rabbit 17.—Weight, 1,580 gm.; Jan. 19, 1.5 cc of red corpuscles of the hen, and Jan. 25, 0.5 cc + 1 cc were injected into the vein. After the injection of 1 cc the anaphylactic shock began, but the animal recovered. Feb. 4, hemolytic experiment: 0.05 cc, complete hemolysis; 0.01 cc, incomplete hemolysis; 0.005 cc, no hemolysis. Feb. 4, 1.75 cc of red corpuscles of sheep injected into the vein with no pathologic effect.

Rabbit 18.—Weight, 1,530 gm.; Jan. 19, 1.5 cc; Jan. 15, 0.5 + 1 cc of red corpuscles of the hen were injected intravenously. Feb. 4, hemolytic experiment: 0.002 cc, complete hemolysis; 0.0016 cc, and 0.0014 cc, complete hemolysis; 0.0012 cc, incomplete hemolysis; 0.001 cc, slight hemolysis; 0.0008 cc, no hemolysis. Feb. 4, 1.5 cc of red corpuscles of sheep injected into the vein. After 20 seconds the anaphylactic shock began, and the animal died after 2½ minutes. No thrombi were found in the heart.

Rabbit 19.—Weight, 2,000 gm.; Jan. 25, 1.5 cc, and Feb. 4, 0.5 cc + 1 cc of red corpuscles of the hen were injected into the vein. Feb. 12, hemolytic experiment: 0.01 cc and 0.005 cc, complete hemolysis; 0.003 cc, slight hemolysis; 0.0025 cc, no hemolysis. Feb. 12, 0.5 cc + 1 cc of red corpuscles of the hen were injected into the vein. Feb. 21, hemolytic experiment; same result as on Feb. 12. The experiment of agglutination with red corpuscles of the hen gave the same result as in rabbit 20. Feb. 21, 1.5 cc of red corpuscles of sheep injected intravenously with no pathologic effect. After 15 minutes, 1.5 cc of red corpuscles of the hen injected into the vein; immediately after the injection the anaphylactic shock began, and the animal died after 3 minutes.

Rabbit 20.—Weight, 2,150 gm.; Jan. 25, 1.5 cc; Feb. 4, 0.5 cc + 1 cc, and Feb. 12, 0.5 cc + 1 cc of red corpuscles of the hen were injected into the vein. Feb. 27, hemolytic experiment: 0.01 cc and 0.005 cc, complete hemolysis of red corpuscles of sheep; 0.0025 cc, incomplete hemolysis; 0.002 cc, slight hemolysis; 0.0012 cc, no hemolysis. Experiment of hemagglutination with red corpuscles of the hen: 0.001 cc, 0.0002 cc, and 0.0001 cc, complete agglutination (compact sediment of red corpuscles forming a clot resistant to shaking); with red corpuscles of sheep: 0.01 cc, no agglutination. Feb. 27, 1.5 cc of red corpuscles of sheep injected into the vein with no pathologic result; 15

minutes later 1.5 cc of red corpuscles of the hen injected intravenously; after 25 seconds the anaphylactic shock began, which lasted for 1½ minutes; then the animal recovered.

Rabbit 21.—Weight, 1,870 gm.; Feb. 5, 1.5 cc, and Feb. 17, 0.5 cc + 1 cc of red corpuscles of the hen were injected into the vein. Feb. 27, hemolytic experiment with red corpuscles of sheep: 0.01 cc, 0.005 cc, and 0.003 cc, complete hemolysis; 0.0025 cc and 0.002 cc, almost complete hemolysis; 0.0016 cc, no hemolysis. Hemagglutination with red corpuscles of the hen: 0.001 cc, 0.0002 cc, and 0.0001 cc, complete agglutination; with red corpuscles of sheep: 0.01 cc, positive agglutination; 0.005 cc, negative agglutination. Feb. 27, 1.5 cc of red corpuscles of sheep injected into the vein; no pathologic phenomena; 15 minutes later 1.5 cc of red corpuscles of the hen injected intravenously; after 10 seconds the anaphylactic shock began, and after 6 minutes the animal died.

Rabbit 22.—Weight, 1,650 gm.; Feb. 5, 1.5 cc, and Feb. 15, 0.5 cc + 1.5 cc of red corpuscles of the hen were injected into the vein. Feb. 22, hemolytic experiment: 0.002 cc and 0.0012 cc, complete hemolysis; 0.001 cc, slight hemolysis; 0.0008 cc, no hemolysis. Feb. 22, 1.5 cc of red corpuscles of sheep injected into the vein; after 20 seconds the anaphylactic shock began, and after 8½ minutes the animal died.

Rabbit 23.—Weight, 1,750 gm.; Feb. 5, 1.5 cc, and Feb. 12, 0.5 cc + 1.5 cc of red corpuscles of the hen were injected into the vein. Feb. 19, hemolytic experiment with red corpuscles of sheep: 0.005 cc, complete hemolysis; 0.003 cc, slight hemolysis; 0.0025 cc, no hemolysis. Hemagglutination with red corpuscles of the hen: 0.001 cc, 0.0002 cc, and 0.0001 cc, complete agglutination; with red corpuscles of sheep: 0.01 cc, no agglutination. Feb. 19, 1.5 cc of red corpuscles of sheep injected into the vein with no pathologic result. After 15 minutes, 1.5 cc of red corpuscles of the hen injected into the vein; after 30 seconds the anaphylactic shock began, which lasted for 1 minute, then the animal recovered.

When the animal dies some hours after the anaphylactic test, death is generally preceded by progressive coma: immediately after the injection the rabbit sometimes looks normal, but soon afterward it shows symptoms of extreme weakness; in the majority of cases the extremities are stretched out and in a state of paresis; the animal lies flat on the ground (in the posture of a seal). At the beginning, the rabbit can be forced to move, but gradually extreme prostration develops; the temperature falls progressively. Sometimes death is preceded by convulsions of the entire body, similar to those of the anaphylactic shock; in these cases the animal dies in the position of opisthotonos, the head thrown back at right angles to the body.

The phenomena of heterogeneous cellular anaphylaxis described here may serve as a basis for the solution of the more general problem whether the cellular anaphylaxis exists. The school of Friedberger⁴ questions the existence of cellular anaphylaxis as an independent

⁴ Schiff, F., and Moore, H. F.: *Zeitschr. f. Immunitätsf.*, 1914, 22, p. 618.

phenomenon; they believe that the sensitization of the animal, as well as the anaphylactic shock, is due, not to the red corpuscles, but to the traces of serum that may still adhere to the washed suspension of red corpuscles. My experiments show that cellular anaphylaxis does exist; it is possible that some traces of serum are introduced with the red

TABLE 1*
RESULTS OF ANAPHYLACTIC TESTS

Rabbit No.	Quantity of Hemolysins against Red Corpuseles of Sheep the Day of Anaphylactic Test												Result of Anaphylactic Test
	0.01	0.005	0.003	0.0025	0.002	0.0016	0.0014	0.0012	0.0011	0.001	0.0008	0.0006	
1	C.	C.	C.	C.	C.	..	I	..	N.	Died after 11 hours
2	C.	C.	C.	C.	C.	C.	C.	C.	C.	Al.C.	N.	N.	Anaphylactic shock; died after 2.25 minutes
3	C.	C.	C.	I.	I.	I.	I.	I.	I.	I.	Sl.	N.	Anaphylactic shock; died after 7 minutes
4	C.	C.	C.	C.	C.	Al.C.	I.	N.	Died after 2 hours
5	C.	..	Sl.	N.	Alive; no pathologic phenomena
6	C.	C.	C.	C.	C.	C.	..	I.	Sl.	N.	Died after 10 hours
7	C.	C.	C.	C.	C.	C.	C.	C.	C.	Al.C.	N.	..	Died after 4 hours
8	C.	..	Sl.	N.	Alive; no pathologic phenomena
9	C.	Sl.	N.	Died after 6 hours
10	C.	C.	C.	C.	C.	C.	I.	Sl.	N.	Died after 5 hours
11	..	Al.C.	I.	..	Sl.	N.	Alive; no pathologic phenomena
12	C.	Al.C.	Al.C.	I.	..	Sl.	N.	..	Anaphylactic shock; died after 5 minutes
13	C.	Al.C.	..	I.	Sl.	N.	Anaphylactic shock; rabbit recovered
14	C.	I.	Sl.	..	N.	Alive; no pathologic phenomena
15	C.	Sl.	N.	Alive; no pathologic phenomena
16	I.	Sl.	N.	Alive; no pathologic phenomena
17	I.	N.	Alive; no pathologic phenomena
18	C.	C.	C.	C.	C.	C.	C.	I.	..	Sl.	N.	..	Anaphylactic shock; died after 2.7 minutes
19	C.	C.	Sl.	N.	Alive; no pathologic phenomena
20	C.	C.	Sl.	N.	Alive; no pathologic phenomena
21	C.	C.	C.	Al.C.	Al.C.	N.	Alive; no pathologic phenomena
22	C.	C.	C.	C.	C.	C.	C.	C.	..	Sl.	N.	..	Anaphylactic shock; died after 3.7 minutes
23	C.	C.	Sl.	N.	Alive; no pathologic phenomena

* In the table, C. indicates complete hemolysis; I., incomplete hemolysis; Sl., slight hemolysis; Al.C., almost complete hemolysis; N., no hemolysis.

corpuscles during sensitization and the test; yet serum belongs to two species of animals which are far removed, and under these conditions serous anaphylaxis is excluded.

As to the question of the relation between anaphylactic antibodies and hemolysins, it is to be noticed that the animals which offer the picture

of heterogeneous anaphylaxis always possess heterogeneous hemolysins (table 1); and in the majority of cases the anaphylactic phenomena were directly connected with the degree of the hemolytic titer; only in rabbits 9 and 13 was anaphylaxis observed, while the quantity of hemolysins was relatively small (100 hemolytic units in 1 c c). My experiments show that when the hemolytic titer of an animal is not below 0.002, anaphylaxis always takes place; when the quantity of hemolysins is below this limit, anaphylaxis is seldom observed (rabbit 9).

These experiments agree with those of Friedemann,⁵ who affirms the identity of hemolysins and anaphylactic antibodies. Moreover, I believe that hemolysins and anaphylactic shock (anaphylaxis) are two aspects of the same phenomenon—the first takes place *in vitro*, the

TABLE 2
RESULT OF ANAPHYLACTIC TESTS WITH RED CORPUSCLES

Rabbit No.	Hemolytic Titer against Sheep Corpuscles	Titer of Hemagglutination to Hen Corpuscles	Result of the Anaphylactic Test with Red Corpuscles	
			Of the Sheep Heterogeneous Anaphylaxis	Of the Hen Homologous Anaphylaxis
19	0.003	0.0001*	No pathologic phenomena	Anaphylactic shock; died after 3 minutes
20	0.003	0.0001*	No pathologic phenomena	Anaphylactic shock; recovered
21	0.002	0.0001*	No pathologic phenomena	Anaphylactic shock; died after 6 minutes
23	0.003	0.0001*	No pathologic phenomena	Anaphylactic shock; recovered

* I did not determine the limit titer; this was not important for the problem.

second *in vivo*—both require the presence of substances able to change the degree of dispersion; in one case, the antibodies were produced by immunized animals.

Table 1 shows that in eleven cases there was no heterogeneous anaphylaxis in the rabbits whose hemolytic titer was low (in the majority of cases 0.01 or less; only rabbits 19 and 23 had 0.005 and rabbit 21, 0.002). From my point of view, the absence of anaphylaxis after the test with red corpuscles of sheep in these conditions is easily explained: the change of the degree of dispersion in the circulating plasma of the animal was too slight to cause anaphylaxis, because of the small quantity of antibodies. I reported on this phenomenon some time ago.⁶ This explanation is confirmed by a series of complementary experiments I

⁵ *Ibid.*, 1909, 11, p. 591.

⁶ *Jour. Infect. Dis.*, 1918, 22, p. 101.

carried out for that purpose. The red corpuscles of the hen possess, besides the heterogeneous sheep antigen, a specific antigen, which produces after immunization of animals an enormous quantity of hemagglutinins to red corpuscles of the hen. These homologous antibodies are contained in the serum with heterogeneous antibodies, but in far greater quantity. If my theories about the cause of the absence of heterogeneous anaphylaxis in animals possessing but few heterogeneous hemolysins are true, one may infer that the same animals possessing a large quantity of homologous antibodies should show the picture of homologous anaphylaxis immediately after the injection of the homologous antigen (red corpuscles of the hen) into the blood system. My experiments on rabbits 19, 20, 21, and 23 confirmed these views.

The experiments were carried out as follows: I determined the quantity of heterogeneous hemolysins and of homologous hemagglutinins. If the injection of red corpuscles of sheep was not followed by heterogeneous anaphylaxis, another intravenous injection was made after 15 minutes, this time of red corpuscles of the hen. The details of the experiments and their results are given in the protocols and in table 2.

It is seen from Table 2 that all the rabbits were subject to anaphylactic shock after injection of the sediment of hen red corpuscles.

SUMMARY

The experiments show that rabbits immunized with red globules of the hen produced the anaphylactic antibodies against red corpuscles of sheep.

The animals that presented the picture of heterogeneous anaphylaxis always possessed heterogeneous hemolysins, and in the majority of cases the anaphylactic phenomena were directly connected with the degree of the hemolytic titer.

STUDIES OF FUSIFORM BACILLI AND SPIROCHETES

III. OCCURRENCE IN NORMAL WOMEN ABOUT THE CLITORIS AND SIGNIFICANCE IN CERTAIN GENITAL INFECTIONS

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In the study of the smegma of the preputial sac of men, it was noted that fusiform bacilli and spirochetes occurred in 51%.¹ These findings suggested a similar study of the smegma secretions of the female. A review of the literature revealed no reference to the occurrence of fusiform bacilli about the normal female genitalia. Spirochetes, however, have been observed in the normal secretions about the clitoris by Csillage² and in few numbers by Rona³ in 20 normal women. The latter further described the occurrence of spirochetes together with fusiform bacilli in clitoritis areata and ulcerata and in gangrenous ulcers of the genitalia.⁴ Noguchi⁵ obtained a pure culture of spirochetes from a phagedenic ulcer of the vulva and called the organism *Spirochaeta phagedenis*. Our studies were carried out to determine the presence of the fusiform bacillus together with spirochetes in normal female smegma.

The women were dispensary patients, chiefly primipara, who entered for examination during the last months of pregnancy. These women were selected because they were not taking douches and were free from venereal infection. The preputium clitoris was pushed back and with sterile applicator the smegma between the body of the clitoris and the inner lining of the preputium was removed. In 5 instances, the amount of smegma was profuse; in 30, moderate, and scanty in one. Direct smears were made on slides, and some of the smegma was placed in sterile salt solution for cultural purposes. The smears were stained with 10% carbol fuchsin.

Specimens of smegma of 36 women were examined, and spirochetes together with fusiform bacilli were found in 21, or 58%. In many, the

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¹ Jour. Infect. Dis., 1923.

² Arch. f. Dermat. u. Syph., 1898, 46, p. 150.

³ Ibid., 1903, 67, p. 259.

⁴ Verhand. d. Deutsch. dermat. Gesellsch., 1906, 9, 471.

⁵ Jour. Exper. Med., 1912, 16, p. 261.

spirochetes were quite numerous, and in others only a few could be found. Fusiform bacilli when present appeared in relatively few numbers. These organisms seldom predominated. As a rule, they occurred in numbers considerably fewer than the associated coccal and bacillary forms.

The spirochetes appeared variable in size—some small and slender with few undulations, others coarser and longer with 5 to 10 turns. In a few instances, they were in groups; otherwise, they appeared scattered among the other bacteria (fig. 1). They were gram-negative and resembled in their morphologic characteristics the spirochetes observed in male smegma.

The fusiform bacilli varied in their morphology. Usually they occurred as straight or slightly curved rods tapering into sharp or blunted ends (fig. 1). As a rule, they stained uniformly; less often unevenly, appearing granular. Careful staining brought out their weak gram-positive character. Occasionally long, threadlike forms of bacilli occurred, which may have been pleomorphic types of fusiform bacilli. Associated with the fusospirochete organisms were constantly large numbers of cocci usually resembling staphylococci, often short gram-negative bacilli, large coarse gram-positive bacilli and diphtheroids. In a few specimens, yeast cells were found.

Anaerobic cultures of the smegma were made by the Dick plate method in serum agar and in Wright pyrogallic blood agar slants. In a few instances, tall tubes of ascites tissue agar were inoculated. In the presence of fusiform bacilli, the cultures had a distinct putrid odor. The bacilli were found in 8 of 18 specimens as short typical forms, sometimes longer and threadlike. In the Dick plates, the bacilli were few and found only after considerable search as small, irregular, granular colonies. Spirochetes did not appear in these cultures. Many other organisms were present, corresponding in their appearance to those of the aerobic cultures.

Aerobic cultures of the smegma were made on infusion agar, to which one part of human defibrinated blood was added to 9 of agar to determine the nature of the associated flora. Thirty-one of 36 specimens revealed staphylococci, chiefly of the albus type. Colon bacilli appeared in 9, large gram-positive bacilli in 5, diphtheroids in 2 and hemolytic streptococci of the beta type in one.

From our studies it is evident that, as in the preputial sac of the male, in the female we are dealing with a locality in which fusiform bacilli and spirochetes are normally present as saprophytes. It is note-

worthy that, as in the male erosive and gangrenous balanitis appears to be due to fusiform bacilli and spirochetes, in the female an analogous infection due to the same organisms results in erosive clitoritis and vulvitis and noma of the vulva. We have observed one instance of erosive vulvitis in a young woman who presented edema of the labia minora with superficial erosions and a shallow nonindurated ulcer on one labia. Darkfield examination revealed short slender spirochetes and bacilli but no *spirochaeta pallida*. In smears stained with carbol fuchsin, fusiform bacilli, short spirochetes and cocci appeared in moderate numbers.

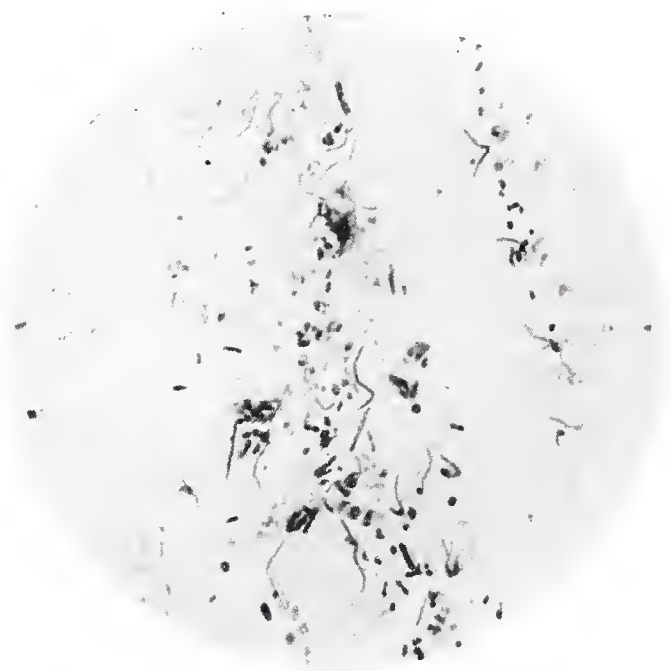


Fig. 1.—Fusiform bacilli, spirochetes and cocci in normal female smegma.

Another instance of the rôle of these organisms in genital infections was illustrated in a specimen of an infected large pedunculated cervical fibroid which was removed from the vagina. The mass extended through the introitus with a green, foul smelling, shaggy surface from which fusiform bacilli, spirochetes and cocci could be demonstrated in abundance. The spirochetes varied in morphology; some were short and slender, others coarse and long. In aerobic cultures, *staphylococcus albus* and in anaerobic cultures short and long thread forms of fusiform bacilli were obtained. Sections stained by the Levaditi method contained spirochetes only in the necrotic surface of the fibroid.

From the study of smears and cultures of normal smegma secretions and certain ulcerative and gangrenous processes we find that there are similarities in the findings suggesting that under certain conditions the organisms normally present may become pathogenic agents. They may also play an important rôle as secondary infecting organisms in specific lesions, and of new growths, such as fibroids and carcinoma of the cervix of the uterus. To prevent such infection, it is therefore important to encourage proper hygiene of the vulva in order to reduce the number of bacteria, particularly in women with lesions about the genitals.

SUMMARY

Fusiform bacilli and spirochetes were found in the normal smegma secretions of 21 of 36 pregnant women. In their morphology, they appear identical with similar organisms in the preputial secretions of men.

Associated with these bacteria were pyogenic organisms, including staphylococci, colon bacilli, diphtheroids and streptococci.

The occurrence of fusiform bacilli and spirochetes with pyogenic bacteria in certain genital lesions would indicate that these organisms may be primary and secondary infecting agents causing ulcerative and gangrenous processes. The presence of these organisms normally would indicate that such processes may result, especially under conditions of lowered general resistance, and do not entirely depend on the introduction of these bacteria from other sources.

BACILLUS WELCHII IN BREAD

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The Welch bacillus, originally described by Welch and Nuttall¹ under the name *B. aerogenes capsulatus*, is probably the best known of the spore-forming anaerobes. It is widely distributed in nature, occurring usually in large numbers in the human intestine and in sewage. It is frequently encountered in water supplies, soil, dust, milk and other foodstuffs and, in addition, has been reported from a great variety of miscellaneous sources.² During the recent war, attention was centered on it as the chief cause of gas gangrene, and as a result the organism has been made the subject of numerous extensive studies, both in this country and abroad, concerning its biology, its biochemical, serologic and toxic properties and especially its relation to wound infection, gas gangrene and the pathology of these infections. It should be understood that *B. welchii* is now usually regarded as representative of a group of similar organisms having in common certain salient characteristics. The recent statement by Kendall, Day and Walker,³ "It seems probable that the Welch bacillus is the type member of either a group of closely related organisms, or of a series of identifiable variants of the same bacillus" expresses our present opinion.

I recently found that a certain commercial "bread starter" or leaven, advocated for the preparation of salt-rising bread, contained large numbers of the Welch bacillus and that this organism was evidently the active agent concerned in the preparation of the bread. As this represented an entirely new phase of the activities of *B. welchii*, and since the use of an organism of this type in an industrial process presented certain unique aspects, it was investigated further and this investigation forms the basis of this report.

At this point an explanation should be given as to what constitutes this particular kind of bread. The term salt-rising bread is a very old one and has been applied to a type of bread in which chance inoculation by the proper gas-forming micro-organisms is depended on to secure sufficient gas production to cause the dough to rise. Ordinary bakers' yeast is not used. Flour usually contains a variety of bacteria and yeasts in considerable numbers so that when mixed with water gas will slowly develop. The dependence on chance inoculation for

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¹ Bull. Johns Hopkins Hosp., 1892, 3, p. 81.

² Simonds, J. P.: Monograph No. 5, Rockefeller Inst. for Med. Res., 1915.

³ Jour. Infect. Dis., 1922, 30, p. 141.

the proper gas-producing organisms frequently resulted in failure of the dough to rise and produced a heavy, sour loaf. The large proportion of failures to secure a palatable loaf has probably been responsible for the limited preparation of this type of bread, at least on a commercial scale. The advantage which is claimed for the particular "starter" in question is that it insures a constant inoculum of a gas-forming bacterium and, therefore, its use on a large scale in bakeries has been advocated by its originator.

The "starter" is a coarse white powder which contains, in addition to the inoculation of micro-organisms, starch and certain alkaline salts. Directions which accompany the "starter" call for its addition to boiling hot milk. This mixture is to be held over night (10 to 12 hours) in a warm place, when it has become light and frothy because of vigorous evolution of gas. It is then added to flour and hot water to prepare a "sponge" which is allowed to rise, after which more flour, hot water and other ingredients are added to form the dough which is made into loaves, again allowed to rise, and baked.

Since Buchanan⁴ states that the bacteria frequently concerned in this type of panary fermentation are organisms of the *B. coli* and *B. lactis-aerogenes* groups, the first examination of the commercial "bread starter" was directed toward the detection and estimation of the numbers of the colon-aerogenes group. Repeated examinations, however, both by direct plating and after preliminary enrichment in lactose broth, yielded organisms of this group in only one instance. Abundant gas formation occurred in the lactose broth tubes, but the organism responsible for this could not be found on Endo plates streaked from these tubes. The rare occurrence of the colon-aerogenes group in the starter suggested that it was present only as an accidental contamination and not as the active agent in the fermentation. Also, the directions for use which accompanied the starter indicated that the organism could not be a member of the colon-aerogenes group since they called for the addition of the starter to boiling hot milk, a procedure which would be expected to eliminate non-spore-forming organisms. During the course of subsequent work, several publications⁵ on salt-rising bread were obtained which state that the organism employed is a spore-forming, rod-shaped bacillus.

The vigorous gas production in lactose broth and the failure to grow on aerobic Endo plates, together with the appearance of milk

⁴ Bacteriology for Students in General and Household Science, 1921, p. 266.

⁵ Kohman, H. A.: U. S. Patent, 1915, No. 1,149,839; Sc. Am. Suppl., 1917, 84, p. 212.

after incubation with the starter and the morphology of the predominating organism, suggested the presence of members of the *B. welchii* group. Subsequent examinations have shown the presence in considerable numbers of an organism which when isolated and subjected to further study was found to be *B. welchii*.

In the course of this work, when testing for the presence of the Welch bacillus in the "starter" and in bread, it became necessary to adopt a routine procedure which would be as simple as possible and permit the examination of a number of samples in a short time. For this purpose chief reliance was placed on the typical stormy fermentation in milk. This was supplemented by the Gram stain to show the stout, short gram-positive rods and also by occasional tests for motility made in hanging drop preparations. These observations on morphology and motility are necessary to distinguish *B. welchii* from several other anaerobes, especially *B. butyricus*, which to a certain extent may produce a stormy fermentation of milk. Hall⁶ states that *B. welchii* is the only nonmotile anaerobe producing stormy fermentation in milk and that *B. butyricus* is differentiated from *B. welchii* by its morphology and motility. Two strains of *B. butyricus* studied by him are said to have given rise to considerable gas with a tardy coagulation in milk. In a personal communication he has informed the writer that *B. butyricus* is frequently as active in milk as *B. welchii*. Heller⁷ states that most species of the genus *Clostridium* of her classification of which the type is *Clostridium butyricum*—cause stormy fermentation of milk. It is believed that the routine procedure as outlined was sufficient to determine the presence of the Welch bacillus and at the same time to distinguish it from *B. butyricus*, with which it might be confused.

DETERMINATION OF NUMBERS OF *B. WELCHII* IN THE "STARTER"

The numbers of *B. welchii* in the "starter" were determined by preparing a series of dilutions in sterile milk. A 1 gm. sample of the starter was weighed with aseptic precautions and transferred to a tube containing 9 c.c. of hot milk. This was well shaken and a series of decimal dilutions were made by transferring successively 1 c.c. to tubes containing 9 c.c. of milk. Owing to the coarse granular nature of the starter an even suspension could not be obtained, and the results are, therefore, merely an approximation of the numbers actually

⁶ Jour. Infect. Dis., 1922, 30, p. 445.

⁷ Jour. Bacteriol., 1922, 7, p. 1.

present. An effort was made to obtain more accurate results by thoroughly grinding the starter before preparing dilutions. However, results obtained by this procedure were no different from the others. The milk tubes, representing the various dilutions of the starter, were then incubated at 37 C. in an anaerobic jar. Conditions suitable for growth were secured by exhausting the air with a vacuum pump and then bringing together pyrogalllic acid and a 10% sodium hydroxide solution. All milk cultures exhibiting the typical stormy fermentation were stained by the Gram method, and motility tests were made on the higher dilutions. The typical vigorous stormy fermentation, accompanied by short, stout nonmotile gram-positive rods was recorded as positive for *B. welchii*. A number of different determinations on several samples of the commercial bread starter gave similar results. *B. welchii* was present in the 1:1,000 dilution in all but one instance, when it was found in 1:100 dilution. The numbers of *B. welchii* present in the starter were estimated, therefore, at approximately 1,000 per gram.

The addition of this starter to milk, followed by overnight incubation, as called for in the directions for use, results in a light frothy mass which was found to contain large numbers of *B. welchii*, usually from 1,000,000 to 100,000,000 per gram. This is then used in the preparation of the salt-rising bread. Since it is evident that large numbers of this organism are incorporated in the dough before baking, it was deemed of interest to determine whether or not the Welch bacillus could be found in the finished loaf. Several bakeries in the city of Washington, D. C., regularly used this particular "starter," and accordingly afforded an opportunity for examination of the salt-rising bread loaves, as placed on sale.

NUMBERS OF *B. WELCHII* IN THE FINISHED BREAD

The following procedure was employed to determine the numbers of *B. welchii* in the finished bread. Known and varying quantities of bread, both from the interior of the loaf and from the crust, were transferred with all possible aseptic precautions to tubes of skim milk. These were not heated after receiving the bread samples, since it was desired to include in the total number the vegetative cells of *B. welchii*, should any of these survive the baking. The milk tubes containing the bread samples were then incubated at 37 C. in anaerobic jars from which the air was exhausted by an electric pump. Final results were not recorded until after 72 hours at 37 C. In the vacuum secured in this

way *B. welchii* produces the typical stormy fermentation with great violence. The casein coagulum is riddled with holes, or it may be entirely pushed up and out of the liquid portion of the culture. The evolution of gas during the first stages of fermentation is vigorous, giving a boiling, frothy appearance to the milk tubes.

Loaves of ordinary bread made with yeast were included as controls for comparison with the salt-rising bread. The occurrence of *B. welchii* in salt-rising bread, as contrasted with ordinary yeast bread, is summarized in table 1. The Welch bacillus was found repeatedly in the interior of the salt-rising loaf, even when small quantities of bread were examined. Thus, of 92 one one-hundredth gram samples, 85 or 92.4% contained the Welch bacillus, while 96.2% of the 0.1 gm.

TABLE 1
COMPARISON OF THE OCCURRENCE OF *B. WELCHII* IN SALT-RISING BREAD AND IN
ORDINARY YEAST BREAD

Quantity of Bread in Individual Samples Examined, Gm.	Interior of Loaf: Number of Samples		% Positive	Exterior, Brown Crust: Number of Samples		% Positive
	+	0		+	0	
Salt-rising bread (26 loaves)						
5.0.....	11	0	(100)	—	—	(—)
1.0.....	57	0	(100)	8	38	(17.4)
0.1.....	75	3	(96.2)	3	47	(6.0)
0.01.....	85	7	(92.4)	0	10	(0.0)
Yeast bread (22 loaves)						
5.0.....	3	37	(7.5)	—	—	(—)
1.0.....	2	51	(3.8)	11	34	(24.4)
0.1.....	0	24	(0.0)	2	40	(4.8)
0.01.....	—	—	(—)	—	—	(—)

samples yielded *B. welchii*. Larger quantities of bread, 1.0 and 5.0 gm., gave uniformly positive results. In decided contrast to this were the results obtained on examination of ordinary yeast bread. Of 40 five gram samples from the interior of the loaves, only 3, or 7.5%, showed the presence of *B. welchii*. Smaller samples of the bread resulted in fewer findings of the Welch bacillus, i. e., 3.8% of the 1.0 gm. samples were positive, while all of the 0.1 gm. samples were negative. This decided difference in the numbers of *B. welchii* present was not apparent on examination of the bread crust, for *B. welchii* was found on 17.4% of the 1.0 gm. samples of salt-rising bread crust and on 24.4% of the 1.0 gm. samples of crust of ordinary yeast bread. Smaller quantities of crust naturally yielded *B. welchii* a fewer number of times. The infrequent occurrence of the Welch bacillus on the browned crust of the salt-rising bread is noteworthy in contrast to its frequent occurrence

in even small quantities taken from the interior of the loaf. Since the proportion of positive findings for *B. welchii* on the crust of the salt-rising bread was no greater than those from yeast bread, the organisms here evidently represent contamination due to handling and dust in the shops and during transportation to the laboratory. The bread in question was not wrapped but lay exposed on racks and on the counter. In a study of the contamination of the surfaces of bread loaves as kept under various sanitary conditions, Howell⁸ found a correlation between the total count, the presence of streptococci and *B. coli* and the sanitary condition under which the loaves were kept. The counts were much less on wrapped than on unwrapped bread.

Through the foregoing examination the milk tubes, after receiving the bread sample, were not heated, as it was deemed advisable to include both spores and vegetative cells, should any of the latter be

TABLE 2

PRESENCE OF *B. WELCHII* IN SALT-RISING BREAD AS SHOWN BY UNHEATED AND HEATED SAMPLES

Quantity of Bread in Individual Samples from Interior of Loaf, Gm.	Unheated: Number of Samples		% of Samples Positive	Heated, 83-85 C. for 15 Min.: Number of Samples		% of Samples Positive
	+	0		+	0	
0.1.....	25	0	(100)	24	0	(100)
0.01.....	38	4	(90.5)	40	3	(93.0)

present. To determine whether the *B. welchii* found in such large numbers in the interior of the loaves of salt-rising bread were present as spores, a series of examinations were made using duplicate sets, one of which was heated to 83-85 C. for 15 minutes, while the other was left unheated. Incubation was carried out in an anaerobic jar as previously described; 0.1-gram and 0.01-gram samples of salt-rising bread were used. The results given in table 2 indicate that the organism is present in the spore form, since heating the samples did not decrease the proportion of positive findings.

CULTURAL AND BIOCHEMICAL PROPERTIES

Since an organism of the *B. welchii* type was found to be present in large numbers in the bread "starter" and also in the interior of the finished loaves of salt-rising bread, it was imperative to isolate the organism and subject it to a comparative study with an authentic strain

⁸ Am. Jour. Pub. Health, 1912, 2, p. 321.

of the Welch bacillus. Altogether 4 bread cultures were obtained, 2 from samples of the starter and 2 from the interior of loaves of salt-rising bread which had been prepared from the starter. The method of isolation consisted in rapid successive transfers in milk, followed by dilution in deep tubes of beef infusion agar containing 0.1% of dextrose. The process of picking from well isolated colonies was carried through several times before further study of the culture. For comparison with the strains used in bread preparation a culture of *B. welchii* was secured from the Army Medical School. This had been obtained originally from an infected war wound and was designated as the "Silverman" strain.

Morphologically, the four bread cultures and the Silverman strain appeared as short thick gram-positive rods, usually occurring singly, but occasionally with a few arranged in pairs end to end. A 24-hour culture of the Silverman strain in 0.1% dextrose beef infusion broth showed a few slightly curved or crescent-shaped forms. Motility was never observed in any culture. In deep tubes of beef infusion 1.5% agar, the colonies of all cultures were disk-shaped and compact but spreading in a thin film when the agar was torn by evolution of gas. The typical stormy fermentation of milk was produced by all the cultures. In the absence of the proper degree of anaerobiosis, clotting of the casein with little or no gas formation occurred, as noted by Simonds.² In chopped meat medium a dense uniform turbidity with evolution of gas appeared within 24 hours. Eventually, the heavy growth settled to the bottom leaving a clear supernatant fluid. The meat was apparently unchanged with no evidence of digestion after standing for several weeks both at 37 C. and at room temperature.

Fermentation tests with various sugars and alcohols were made in deep tubes of beef infusion broth covered with a layer of solid paraffin. The various test substances were sterilized separately in 10% solution and added aseptically to tubes of the broth in sufficient amount to give a final concentration of 1%. Fermentation was determined by an increase in the hydrogen-ion concentration over that shown by inoculated control tubes of plain broth and also by recording the gas formation. The hydrogen-ion concentration was determined colorimetrically using the indicators of Clark and Lubs.⁹ Dextrose, lactose, sucrose, maltose and inulin were fermented vigorously by the 4 bread cultures and by the Silverman strain. Glycerol also was fermented by all of the cultures, although the 4 bread strains usually exhibited

⁹ Jour. Bacteriol., 1917, 2, p. 1.

a rather feeble fermentation and did not attain the H-ion concentration reached by the Silverman strain. Mannite and dulcitol were not attacked. In the presence of a fermentable sugar, all of the cultures grew with a dense uniform turbidity which after from 3 to 4 days at 37 C. began to settle to the bottom, eventually leaving a clear fluid above. In all points studied the cultures obtained from the starter and from the bread resembled the typical Welch bacillus as described in the literature and as exemplified in the Silverman strain which had come from a pathologic source.

PATHOGENICITY

For virulence tests 24-hour cultures in beef infusion broth containing a piece of cooked meat were employed. The infusion had not been fermented free from muscle sugars. One and 0.1 c.c. quantities of these cultures were injected into the right thigh muscle of guinea-pigs, with the following results:

Silverman Strain.—1.0 c.c.: The guinea-pig showed symptoms of illness in 5-6 hours and died in less than 18 hours. Entire thigh greatly swollen. Necropsy showed emphysema and edema of thigh muscles with slightly bloody serous infiltration of muscles and subcutaneous tissues. Bloody serous infiltration extended over the entire region of thigh and flank and over the abdomen about midway to the axilla. Thigh muscles pale pink and soft. No putrefactive odor. Peritoneal cavity fairly dry, no effusion of liquid and no inflammation.

0.1 c.c.: Guinea-pig died in less than 18 hours. Presented the same picture as that given above, with the exception that the serous infiltration did not extend quite so far over the abdomen.

Starter No. 1.—1.0 c.c.: Guinea-pig quite sick after 24 hours. Lesion at point of injection; entire thigh and flank swollen and tender. The animal gradually recovered. The hair over the affected region fell out, and an open ulcer was formed which eventually healed forming a scar.

0.1 c.c.: After 24 hours the animal was fairly lively, with a moderate swelling only at point of injection. The swelling disappeared after several days. There was no loss of hair and no ulcer at the site of inoculation.

Bread Strain No. 1.—1.0 c.c.: Animal somewhat sick after 24 hours. Swollen area over whole thigh and flank. Condition of animal gradually improved. There was loss of hair over the affected region and formation of an extensive scar.

0.1 c.c.: Animal very little affected. No general disturbance and only a slight swelling at point of inoculation. Swelling disappeared without loss of hair or formation of surface ulcer.

Virulence tests were also made with additional cultures of *B. welchii*, starter No. 2 and bread No. 2. The results were quite similar to those given for starter No. 1 and bread No. 1 and are not given in detail here.

It is evident from these tests that the Silverman strain of *B. welchii*, which had been obtained from a human wound, was highly virulent, while the strain used in the preparation of the bread—as represented by 2 cultures from the starter and 2 from the bread—possessed only a low grade of virulence, insufficient to cause the death of guinea-pigs following intramuscular injection of 1.0 c.c. amounts of culture.

EXPERIMENTAL PREPARATION OF BREAD

All of the foregoing results indicate that in the cultures from the starter and the salt-rising bread, we are dealing with a typical *B. welchii* of low virulence. Since at first glance it may be considered unlikely that *B. welchii*, as obtained from pathologic material, could multiply under the conditions imposed by bread making, some further experiments were carried out to determine this point. An attempt was

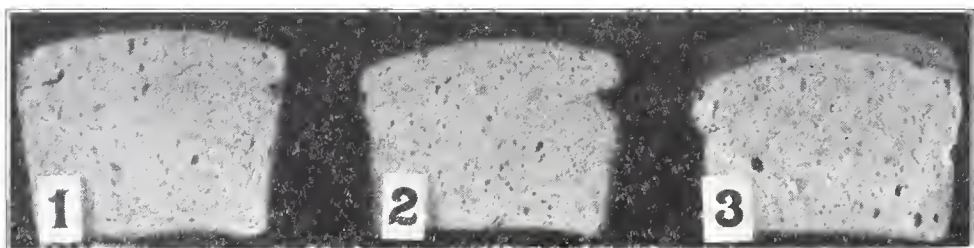


Fig. 1.—No. 1, prepared by use of the commercial starter; Nos. 2 and 3, prepared by substitution of a known *B. welchii* culture for that contained in the starter.

made to prepare similar loaves of bread by the use of the Silverman strain, which had a definite history of wound infection. This organism was substituted for that in the commercial starter by first autoclaving the starter and then adding to this sterilized starter a young culture—18 to 24 hours—of the Silverman strain. Culture tests in milk showed the autoclaving to be sufficient for the destruction of the “gas bacillus” originally present in the starter. The purpose of adding the substituted culture to the starter, instead of using a broth or milk culture directly in the preparation of the bread, was to insure the presence of the alkaline salts which constitute part of the starter.

The salt-rising bread prepared with the Silverman strain of *B. welchii* compared favorably in size and texture with that prepared from the starter. Apparently the strain of the Welch bacillus from an infected wound was also an efficient bread maker. Figure 1 shows the results of a baking experiment in which one of the loaves “1” was prepared by the use of the commercial starter, while those designated

"2" and "3" were prepared from the sterilized starter to which had been added the Silverman culture.

To gain some idea of the number of *B. welchii* present at different stages during preparation of the bread, samples were taken from time to time and an estimate of the numbers present was made by preparing successive decimal dilutions in milk tubes. The procedure was the same as that described at first in the determination of the numbers in the starter. Results obtained in the preparation of the three loaves—1, 2 and 3—shown in the illustration are given here. The figures represent the highest dilution showing the presence of *B. welchii*.

It is quite evident from these results that the addition of the starter to milk with the overnight incubation results in a profuse

TABLE 3
NUMBERS OF *B. WELCHII* DURING PREPARATION OF SALT-RISING BREAD MADE FROM
COMMERCIAL STARTER AND *B. WELCHII*

	Commercial Starter	B. Welchii (Silverman Strain)	
	1	2	3
(a) Starter plus milk, after standing overnight and just before use.....	100,000,000	10,000,000	10,000,000
(b) Sponge, immediately after adding the above to flour and hot water.....	1,000,000	1,000,000	1,000,000
(c) Sponge, after rising (incubation of 1½ to 4 hours)	100,000,000	10,000,000	10,000,000
To show the presence of spore forms only:			
(a) Heated	1,000	100	100
(b) Heated	1,000	10	10

growth of the Welch bacillus. The next step in the procedure causes a decrease in the numbers, due presumably to the addition of flour and hot water, although multiplication continues, for after incubation of the "sponge" for several hours an increase in numbers is apparent [see (b) and (c)]. As would be expected, the vast majority of the organisms are in the vegetative stage, with relatively few spore forms. The results previously given in table 2 show that *B. welchii* is present in the spore form only in the interior of the finished loaf as turned out by the bakeries in the city of Washington. Whether or not the spores are present in the dough in sufficient quantities to account for the uniform findings in even small amounts of the bread is perhaps open to question, especially in the case of the loaf designated as "2" in the foregoing experiment. Several salt-rising loaves prepared experimentally in the laboratory did not give quite as high a percentage of positive findings for *B. welchii* as those procured from the several bakeries.

DISCUSSION

All the evidence gathered in this investigation of the salt-rising "starter" and the bread prepared by its use points to the conclusion that an organism of the Welch bacillus type is the one relied on to secure gas production and the successful preparation of the bread. The description of the organism used in the "starter", or leaven, which is given in the patent⁵ does not agree entirely with that of the Welch bacillus or with the organism isolated from the "starter" and bread in the present investigation. An examination of the cultural properties mentioned in the patent gives the impression that an impure culture of the Welch bacillus was used, although the description is not complete enough to establish the identity of the organism.

The use of an organism of the *B. welchii* type in a commercial process¹⁰ is believed to be unique and at the same time presents certain public health considerations. Shall its use be considered as dangerous and undesirable? The association of the *B. welchii* type with wound infections is well known. It is also recognized that the organism is more or less widely distributed in nature and is a common inhabitant of the intestinal tract of man. It has been claimed by some that it is the causative agent in certain diarrheas, with which it is usually associated in large numbers. This is still a disputed point, and until more definite evidence is brought forward the question as to the use of this organism in bread preparation should perhaps be left open. Since the Welch bacillus occurs frequently in milk and is undoubtedly consumed with other raw food products, the point may be raised that there would seem to be no objection to its consumption in bread, at least at the present state of our knowledge. It is reasonable to assume that with our increasing knowledge of the metabolic properties of micro-organisms, various species will be adopted and play an increasing part in industrial processes, so that the safest course would seem to lie in the choice of organisms whose use is beyond question from the standpoint of public health.

SUMMARY

A commercial bread starter recommended for the purpose of securing a constant inoculum of a gas-forming bacterium in the preparation of salt-rising bread, was found to contain organisms of the *B. welchii* type in numbers of about 1,000 per gram.

¹⁰ Literature received with the starter states that it is sold exclusively to the baking trade and not for household use.

The addition of this "starter" to milk followed by an overnight incubation in a warm place, as called for in the directions for use, results in a light, frothy, gaseous mass. The predominating organism was found to be the Welch bacillus, which was present in numbers of 1,000,000 to 100,000,000 per gram of material.

Loaves of salt-rising bread prepared by several bakeries using the starter in question contained spores of *B. welchii* in considerable numbers. Small quantities of bread from the interior of the loaves yielded the gas bacillus in almost every instance, in decided contrast to the results secured from the interior of the loaves of ordinary yeast bread in which the Welch bacillus was found rarely.

Several cultures of the organism isolated from the starter and from the baked loaves were found to be in agreement in morphologic, cultural, and biochemical properties with a strain of *B. welchii* obtained originally from a wound and also with the published descriptions of the Welch bacillus.

The cultures obtained from the bread possessed only a low grade of virulence for guinea-pigs, while the type *B. welchii* originally obtained from a wound and used for comparison was highly pathogenic.

All available evidence indicated that the Welch bacillus incorporated in the "starter" is the active agent concerned in the preparation of the salt-rising bread. This was substantiated by the fact that on substitution of an authentic *B. welchii* strain with a history of wound infection for the strain in the bread starter, loaves of salt-rising bread were prepared comparable to those obtained by the use of the starter.

BACTERIOSTATIC ACTION OF DYES ON STREPTOCOCCUS VIRIDANS AND PNEUMOCOCCI

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This work was undertaken with the hope of finding some dye which could be used in a medium for the differentiation of the pneumococcus and streptococcus groups of bacteria and with the object of determining the relation between chemical constitution and inhibitory power toward these organisms.

The bacteriostatic action of certain dyes on bacteria was observed as early as 1887, but, with the exception of some work by Stilling in 1890, no really systematic study was attempted until the work of Churchman¹ in 1912 and that of Simon and Wood² in 1914. An historical summary is given by Simon and Wood. Churchman used only one dye, gentian violet, and maintained that its inhibitory action could be substituted for the Gram stain as a means of classifying bacteria, and that the accuracy obtained was far greater. The studies of Simon and Wood were extensive, although in their work only one concentration of each dye was used, namely, 1:100,000. They studied the relation of chemical constitution to inhibitory power of the dyes—the point of view which we have taken in our problem. It was found by them that the triamino-triphenyl-methane dyes had the greatest bacteriostatic action, and that this was dependent not only on the triphenyl methane grouping, but also on the amino auxochrome group. Acid dyes had little effect as compared with basic dyes. The action of substituted alkyl groups was uncertain. Krumwiede and Pratt³ found that the streptococcus-pneumococcus group was more resistant to dyes than other gram-positive bacteria. Among later papers should be mentioned those of Kligler,⁴ who also found triphenyl-methane dyes the most active, of Graham-Smith⁵ and of Binger.⁶ A number of other papers have been published during the last few years, but all have dealt with some special dye or combination of dyes and not with the more general phases of the problem.

TECHNIC

We have used five strains of *Streptococcus viridans*, the 3 type pneumococci and one type 4 pneumococcus. The medium employed was Hiss serum-water veal infusion agar. Water solutions of the dyes

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¹ Jour. Exper. Med., 1912, 16, p. 221; p. 822; 1913, 17, p. 373.

² Am. Jour. Med. Sc., 1914, 147, pp. 247 and 524.

³ Jour. Exper. Med., 1914, 19, pp. 20 and 501.

⁴ Ibid., 1918, 27, p. 462.

⁵ Jour. Hyg., 1919, 18, p. 1.

⁶ Jour. Infect. Dis., 1919, 25, p. 277.

were added to measured amounts of the medium in tubes so as to obtain dilutions of the dyes varying from 1:1,000 to 1:50,000 (in some cases as high as 1:1,000,000), the medium then poured into a Petri dish and allowed to harden. The surface was then inoculated from the water of condensation in a 24-hour blood agar slant of the culture to be tested and spread by means of a bent glass rod. The 9 strains of bacteria were used with each dilution of the dye. Observations were made after 24 and 48 hours' incubation at 37 C.

DYES

The following is a list of the dyes tested, together with their classification as far as could be determined.

Nitro Compounds: Martius yellow.

Azo Dyestuffs: Monoazo dyes: aminoazo compounds: chrysoidin, vesuvin, tropäolin 00, methyl orange, methyl red.

Monoazo Dyes: Oxyazo compounds: orange G, tropäolin 000.

Diazo Dyes: scarlet red, Bismarck brown.

Triphenylmethane Dyestuffs: Diamino compounds: malachite green, brilliant green.

Triamino compounds: magenta, methyl violet B, crystal violet, methyl green, night blue, dahlia, gentian violet.

Trihydroxy compounds: rosolic acid.

Oxyketone Dyestuffs: alizarine.

Pyronine Dyestuffs: pyronines: pyronin.

Phthaleins: fluoresceïn, eosine, erythrosine, phenol sulphonephthalein.

Diphenylamine Dyestuffs: thiazines: thionin, toluidin blue, methylene blue.

Azines: neutral red, janus green, safranin, nigrosin, indigo.

Formulae not found: carmin red, potassium indigo sulphate, axolitmin, fluorochrome, carmalum, hematoxylin.

RESULTS

Of the 41 dyes used in our experiments only 16 showed bacteriostatic action in dilutions of 1:1,000 or greater. The following table shows the highest dilution at which complete inhibition was obtained with these dyes.

Of the 16 dyes which showed bacteriostasis, 8 belonged to the triphenyl-methane group and 6 to the diphenylamines. Of the two remaining, one was an azo and the other a diazo compound. Of the 8 triphenyl-methanes, 6 were triamino compounds and 2 diamino. There was no difference between the action of the dyes on the strains of *Streptococcus viridans* and those of the pneumococci.

A survey of the chemical structure of these dyes shows that with one exception all contained 3 benzol rings either attached to a single

carbon atom as in triphenyl-methane, or connected as in anthracene. In some cases, a ring carbon atom is substituted by nitrogen. Vesuvium is an exception, but the formula of this dye appears to be uncertain. In addition to the 3 benzol rings, all these active dyes contain 2 or more amino groups. The hydrogen atoms in these groups were all substituted with alkyl radicals in those dyes showing the greatest inhibitory action. In most instances, this alkyl radical is the methyl group, but in brilliant green—the most active dye we found—ethyl groups are present. An example of the effect of substituting methyl radicals is shown by comparing thionin, a relatively weak dye, with toluidin blue, which has 10 times the bacteriostatic power of thionin. The two differ only in that toluidin blue contains methyl groups substituted for amine hydrogens.

TABLE 1
BACTERIOSTATIC ACTION OF DYES ON THE PNEUMOCOCCUS-STREPTOCOCCUS GROUP

Dye	Highest Dilution Giving Complete Inhibition	Classification
Brilliant green.....	200,000	Diamino-triphenyl-methane
Gentian violet.....	40,000	Triamino-triphenyl-methane
Crystal violet.....	40,000	Triamino-triphenyl-methane
Vesuvium.....	25,000	Amino monoazo
Methylene blue.....	25,000	Diphenylamine
Safranin.....	25,000	Diphenylamine
Night blue.....	20,000	Triamino-triphenyl-methane
Toluidin blue.....	10,000	Diphenylamine
Neutral red.....	10,000	Diphenylamine
Janus green.....	10,000	Diphenylamine
Magenta.....	10,000	Triamino-triphenyl-methane
Dahlia.....	10,000	Triamino-triphenyl-methane
Methyl-violet.....	10,000	Triamino-triphenyl-methane
Malachite green.....	5,000	Diamino-triphenyl-methane
Bismarck brown.....	1,000	Diazo
Thionin.....	1,000	Diphenylamine

Acid dyes or salts of acid or basic dyes were inert. Illustrations are the phthaleins and methyl green.

The substitution of an hydroxyl radical for a methyl group decreases the inhibitory power.

SUMMARY

The organisms belonging to *Streptococcus viridans* and pneumococcus groups are inhibited by dyes to the same extent. It is therefore not feasible to use any dye for differential purposes.

To have marked bacteriostatic action, a dye must contain 3 benzol rings and 2 or more amino groups in which the hydrogen atoms have been substituted by alkyl radicals.

Acid dyes or salts of acid or basic dyes are practically inert.

WEIL-FELIX REACTION IN ROCKY MOUNTAIN SPOTTED FEVER

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The agglutination of *B. proteus* by the blood serum of typhus fever patients first reported by Weil and Felix in 1915 has proved a valuable aid in the diagnosis of this disease. Although the explanation for this reaction is still a matter of dispute, some investigators claiming that it is due to a secondary infection with *B. proteus* and others holding that it is due to the formation of heterogeneous agglutinins, its use as a diagnostic aid is becoming more and more general.

Rocky Mountain spotted fever, a disease closely resembling typhus fever, is present in some parts of the United States, one of which is Northern California. While the separate entity of these two diseases has been proven beyond doubt, the clinical similarity is so marked that a differential diagnosis is often difficult, Castellani and Chambers¹ stating that, "The distinction between spotted fever and typhus on clinical grounds seem to us impossible." Table 1, taken from Fricks,² will serve to show this great similarity.

Until the discovery of the Weil-Felix reaction, the only laboratory method of differentiating between Rocky Mountain spotted and typhus fevers was by means of animal inoculation. Neill³ showed that the scrotal lesions in guinea-pigs inoculated with spotted fever may also be present in those inoculated with typhus. However, these lesions are less marked in typhus than in spotted fever, so that the test serves to differentiate between the two diseases. The difficulty with animal inoculation is its uncertainty. Unless animals are inoculated with comparatively fresh blood, the infective organism of Rocky Mountain spotted fever may not survive. Ricketts⁴ found the blood of Rocky Mountain spotted fever patients to be infective for 10 to 15 days when kept on ice, and Wolbach⁵ found that it did not lose its infectivity for 6 to 8 days at room temperature. That it may lose its infectivity in

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¹ Manual of Tropical Medicine, 1919, p. 1347.

² Pub. Health Reports, 1916, Suppl. 28.

³ Pub. Health Rept., 1917, 32, p. 1105.

⁴ Jour. Infect. Dis., 1907, 4, p. 141.

⁵ Jour. Med. Res., 1919, 41, p. 1.

less time was shown by some experiments carried on during an investigation of the disease in 1916.⁶ At that time blood was taken from 2 patients in Susanville, one at the height of the febrile period, the other well along into the third week. The specimens were citrated and part of each was immediately injected intraperitoneally into guinea-pigs. The other parts of the specimens were sent to the State Hygienic Laboratory at Berkeley where guinea-pigs were inoculated about 5 days later. The animals inoculated with the fresh blood from each of the patients developed spotted fever. The animals inoculated in Berkeley with the blood from the acute febrile case developed the disease, but those inocu-

TABLE 1
COMPARISON OF CLINICAL SYMPTOMS OF ROCKY MOUNTAIN SPOTTED AND TYPHUS FEVERS

	Rocky Mountain Spotted Fever	Typhus Fever
Incubation Period	3-12 days, usually 4-7	4-14 days, usually 8-12
Onset and early symptoms	Onset rapid; chill or chilly sensation; marked prostration; expression heavy; patient very ill; maximum temperature second to third day	Onset abrupt; chill frequent; prostration marked; expression heavy; patient very ill; maximum temperature second to third day
Eruption	Primary eruption macular-roseolar second to third day followed by petechial eruption which does not disappear on pressure; macules become slightly indurated but never true papules; petechiae may become purpuric and be followed by sloughing	Character and distribution almost similar to Rocky Mountain spotted fever, but generally appears first on thorax; face frequently free
Course of disease	Duration 2-3 weeks; temperature continuous and moderately high throughout the attack, falling rapidly as convalescence begins; slight desquamation	Course similar to that of Rocky Mountain spotted fever
Termination	Recovery slow in severe cases, but without sequelae; followed by persistent mottling and marked immunity	Similar to that in Rocky Mountain spotted fever

lated with the specimens from the more advanced case showed no lesions. Animal inoculation may fail from still another cause, as the blood may be taken so late in the disease that it fails to be infective, probably due to the formation of antibodies (Ricketts and Gomez⁷).

The similarity of Rocky Mountain spotted fever to typhus fever suggested that the Weil-Felix reaction might be present in the former disease also. If the reaction were not present in Rocky Mountain spotted fever, the test would be of value in differentiating between the two diseases. Since this is an agglutination test, it is not subject to the uncertainty of animal inoculation, for delays in transportation would not

⁶ Kelly, F. L.: Calif. State Jour. Med., 1916, 14, p. 407.

⁷ Jour. Infect. Dis., 1908, 5, p. 221.

affect the blood serum, and specimens could be taken late in the disease. Through the courtesy of Dr. W. E. Dozier and Dr. R. W. T. Garner of Susanville, Dr. F. J. Davis of Westwood and Dr. C. M. Tinsman of Adin, specimens of blood were obtained from 9 cases of Rocky Mountain spotted fever. Macroscopic agglutination tests were set up using a strain of *B. proteus* X₁₉ obtained from the U. S. Hygienic Laboratory. The first specimen tested was from a patient in the third or fourth week of the disease. This gave a positive agglutination in a dilution of 1:400, but the only antigen used was the first 24-hour transplant from a stock culture of *B. proteus* X₁₉. As the control was negative, the result was not due to spontaneous agglutination, which sometimes occurs in cultures of *B. proteus*. This same specimen was again tested 2 days

TABLE 2
RESULTS OF WEIL-FELIX REACTION WITH SERUM FROM ROCKY MOUNTAIN SPOTTED
FEVER PATIENTS

Case	Week of Disease	24-Hour Plain Agar	24-Hour Glucose Agar	Formalized Antigen
1	3-4	+ 1/400
1	3-4	—	—	—
2	4-5	—	—	—
3	3-4	—	—	—
4	2-3	—	—	—
5	1-2	—	—	—
6	1-2	—	—	—
7	1-2	—	—	—
8	3-4	—	—	—
9	3-4	—	..	—

later, using as antigens a 24-hour agar culture, a 24-hour glucose agar culture and a formalized antigen. This series of tests was negative. No satisfactory explanation for the difference in results between these tests and the previous one has been found. It may be possible that the culture used in the earlier test, being the first transplant from an old culture, was more easily agglutinated, and that this would have happened with normal serum. In the tests of the other 8 specimens, both a live culture and a formalized antigen were used. All of these tests were negative, as shown in table 2.

CONCLUSIONS

While the series of tests is not large enough to warrant any definite conclusions, it suggests that the Weil-Felix reaction is negative in Rocky Mountain spotted fever and may be of value in differentiating between this disease and typhus fever.

INVASION OF BODY BY BACTERIA FROM INTESTINAL TRACT

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The number and the varieties of organisms which enter the intestine are greatly reduced by the antiseptic action of the gastric juice. Normal stomach contents after an Ewald meal are usually sterile so far as concerns ordinary pyogenic bacteria; yeasts, sarcinae and certain aciduric bacteria are frequently found in stomach contents of normal or increased acidity. Gastric juice (free HCL 20-40) or hydrochloric acid of corresponding strength in a dilution of 1 to 16 will kill hemolytic streptococci in 10 minutes and in higher dilutions up to 1 to 64 in one hour. Other common pathogenic bacteria, such as the colon bacillus and *Streptococcus viridans* show a similar, though somewhat less degree of susceptibility to normal gastric juice. This antiseptic action is not continuously effective throughout the 24 hours, however, for we were able to isolate hemolytic streptococci from the stools of 30% of 85 patients with scarlet fever, although in some instances repeated attempts were necessary.¹ Organisms within the intestine thus may be recent arrivals as well as older residents.

The permeability of the wall of the intestine to small particles and bacteria within its lumen has been extensively studied in the investigation of the intestinal origin of tuberculosis, of pulmonary anthracosis, of conditions affecting the sterility of the stored blood serum, of the route of invasion of certain infectious diseases and in the course of observations on antiperistalsis and on the mechanism of the absorption of fat. Experiments in each of these fields are subject to technical difficulties, and the discordant findings of different investigators have resulted in prolonged discussion. Many of the data are of value in the present problem, in which we desire to know to what extent, under what circumstances and by what route organisms within the intestine may pass through its wall and enter the blood stream.

TUBERCULOSIS

Calmette² and others after him showed that tubercle bacilli may pass through the intestinal wall, often without leaving any demonstrable lesion of the mucous

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¹ Jour. Infect. Dis., 1920, 27, p. 363.

² Ann. de l'Inst. Pasteur, 1905, 14, p. 601.

membrane. He showed that young goats suckled by mothers whose mammary glands had been inoculated with tubercle bacilli, developed extensive tuberculosis of the mesenteric and peribronchial glands. In adult goats to which tubercle bacilli had been fed, there was slight tuberculosis of the mesenteric glands but rapidly developing pulmonary tuberculosis. At necropsy, no lesions of the intestinal mucosa were found. To meet the objection that intestinal lesions might have been present originally and healed, Basset and Carré³ fed suspensions of tubercle bacilli to dogs, killed them after periods of 6 hours to 4 days, and inoculated emulsions of the mesenteric lymph glands into guinea-pigs. None of the guinea-pigs inoculated with material from dogs, killed up to 36 hours after feeding, became tuberculous, but pigs inoculated from dogs killed after 3 or 4 days did develop generalized tuberculosis. Ravenel⁴ (who has reviewed this literature) fed tubercle bacilli to dogs previously purged with castor oil, and was able to demonstrate tubercle bacilli in mesenteric glands or chyle by means of guinea-pig inoculation 3 or 4 hours after the injection of bacilli. No lesions of the intestinal wall of the dogs were detected.

ANTHRACOSIS

The study of the intestinal tract as the route of invasion of the carbon particles which give rise to pulmonary anthracosis, resulted in a number of experiments of interest in the determination of the permeability of the intestinal wall to inert particles. Vansteenberghe and Grysez⁵ claimed to have produced pulmonary anthracosis by the introduction of carbon particles into the intestine, and believed that the carbon particles followed the same route as that outlined by Calmette for tubercle bacilli; that the particles passed into the lymphatics of the intestinal wall, thence to the thoracic duct, the right side of the heart and lungs. While the main contention of Calmette and his pupils that the intestinal route was important in pulmonary anthracosis was not sustained, it was clearly shown by both opponents and advocates of the theory, that after the injection of carbon or vermilion, small particles of these substances were demonstrable in the intestinal walls and in the mesenteric lymph glands.⁶ It appeared also that the degree of pigment deposition was proportional to the amount ingested, and that there was evidence of intestinal irritation in many instances.

McCallum⁷ fed albuminate of iron to necturus which had received no food for thirty months. Eight hours after feeding, portions of the intestine were hardened, cut, and stained. Leukocytes in the lumen and others in the intestinal wall contained iron. Similar experiments on higher animals revealed iron containing leukocytes in the tips of the intestinal villi, in the venules of the villi, and later in the capillaries of the liver and spleen.

EXPERIMENTS ON THE STERILITY OF BLOOD AND SERUM

Desoubry and Porcher,⁸ pupils of Nocard, concluded from their experiments that microorganisms could readily enter the blood from the intestine by way of the thoracic duct. M. Neisser⁹ repeated and amplified their experiments and

³ Compt. rend. Soc. de biol., 1907, 63, p. 272.

⁴ Jour. Med. Res., 1903, 10, p. 460; 1908, 18, p. 1.

⁵ Ann. de l'Inst. Pasteur, 1905, 19, p. 787.

⁶ Montgomery, C. M.; Jour. Med. Res., 1910, 23, p. 111, gives review of literature, criticism of evidence and original experiments in which pigment (carmine) after ingestion was demonstrated in the intestinal wall and in mesenteric glands.

⁷ Jour. Physiology, 1894, 16, p. 268.

⁸ Compt. rend. Soc. de biol., 1895, 48, p. 344.

⁹ Ztschr. f. Hyg. u. Infektionskr., 1896, 22, p. 12.

arrived at the opposite conclusion, maintaining that bacteria whether pathogenic or saprophytic do not regularly enter the body from the intestine, and that instances of infection in this way are exceptional. Basset and Carré¹⁰ agreed with this view, but found that in the presence of an acute enteritis occasional microorganisms were able to pass through the bowel wall and appear in the blood and chyle.

In his discussion of latent infection and subinfection in relation to hemochromatosis and cirrhosis, Adami¹¹ maintained that the entry of organisms, especially colon bacilli, from the intestine into the body must be a not uncommon event, although under normal conditions they are quickly destroyed in the mesenteric lymphnodes, blood, liver and other organs. In terminal and agonal infections, the findings at necropsy of organisms of intestinal origin in distant parts of the body indicates the ease with which invasion may occur when the normal factors of resistance are depressed or absent.

The importance attached in earlier work to the giving of fat with bacteria in feeding experiments was based to a large extent on the belief that fat droplets passed unchanged through the intestinal wall into the lymphatics, and that hence during the digestion of fat a favorable opportunity was afforded for the simultaneous passage of bacteria. While the demonstration that fat absorption consists of hydrolysis of fat in the intestine with absorption of the resulting fatty acids and glycerol and their subsequent synthesis into fat, has done away with the theoretical value of fat droplets as carriers of bacteria, studies of the intestinal wall during fat digestion have shown that there is no rigid cell membrane surrounding the columnar epithelial cells of the villi and that soft bodied cells such as leukocytes occurring between them are able to indent and to work their way through between them (Schaefer, Bloor¹²). The giving of fat with bacteria has other advantages, including the possible protection of bacteria in their passage through the stomach, and the increased ease with which the thoracic duct and its tributaries can be identified when filled with chyle after a fat meal in those experiments in which it is desired to make cultures from the chyle.

THE ROUTES OF INVASION OF INFECTIONS

Thiele and Embleton¹³ made an elaborate series of experiments to determine the points of entry and paths of invasion of the body of bacterial infections. In experiments on the gastro-intestinal tract in which *B. mycoides*, *B. coli* and *B. prodigiosus* were introduced into cats by stomach tube and by needle puncture of the duodenum and ileum after laparotomy, they were unable to demonstrate organisms in the chyle from the thoracic duct, or in the blood. When anthrax bacilli were introduced by stomach tube along with finely divided carmine, the bacilli were found in the lungs and urine by culture 4 hours after feeding. No transport of pigment was observed. Cultures from similarly treated animals 24 hours after feeding showed anthrax bacilli in marrow, lungs and other organs.

They concluded that systemic infections can occur through the buccal mucous membrane (feeding experiments with oats treated with *B. mycoides* in guinea-pigs), and that bacteria can pass through the uninjured mucous membrane of the stomach and intestines in some animals and may pass directly into the chyle to be disseminated by the blood stream.

¹⁰ Comp. rend. Soc. de biol., 1907, 62, pp. 261, 349, 890; 63, p. 272.

¹¹ Jour. Amer. Med. Assn., 1899, 33, p. 1508.

¹² Physiological Reviews, 1922, 2, p. 92.

¹³ Proc. Roy. Soc. Med., 1913-14, 7, p. 69.

David and McGill¹⁴ have reviewed the literature on the relation of the bowel to infections of the kidney. In their experimental study on dogs, they found that after the introduction of large amounts of cultures of *B. coli* by the stomach tube, *B. coli* or a *B. coli*-like organism was found in the urinary tract in 3 of 11 dogs in which preliminary cultures of the urine were sterile, and believed that these organisms probably reached the urinary tract by way of the blood stream. Blood cultures in these animals were sterile. In eight of the dogs, organisms were obtained in culture from the mesenteric glands, in 6 instances, *B. coli*. Obstruction of the large bowel, or traumatism of the bowel, was associated with an increased bacterial content of the mesenteric glands, and urinary infection occurred in one dog.

EXPERIMENTS IN DOGS

We have repeated some of the feeding experiments in dogs, and, after isolating the thoracic duct under anesthesia, we made cultures from the thoracic lymph at intervals of 5 to 10 minutes for from 1 to 3 hours, and from the portal blood and viscera after death. Suspensions of *B. prodigiosus*, *B. pyocyaneus* and *Streptococcus hemolyticus* were used for feeding, and cultures from the chyle, blood and organs were made on blood-agar and plain-agar plates and dextrose bouillon.

Exper. 1.—After isolation of the thoracic duct in the neck under ether anesthesia, the abdomen was opened and 10 cc of a suspension of hemolytic streptococci were injected into the duodenum by a hypodermic needle, and the abdomen at once closed. Cultures of the thoracic lymph were made at 5 minute intervals for 2½ hours. No colonies were found on the inoculated plates after 24 hours' incubation, though in a few instances *Staphylococcus albus*, believed to be a contamination, was found in the bouillon cultures.

Exper. 2.—Using heavy suspensions of *B. pyocyaneus*, 6 dogs were similarly injected with from 8 to 15 cc by hypodermic needle into the duodenum. In one dog, plates inoculated with lymph removed 80 and 105 minutes after injection revealed a few colonies of *B. pyocyaneus*, *B. pyocyaneus* was not recovered in lymph from the remaining 5 dogs, although specimens were removed and cultured over a period of 3 hours.

Exper. 3.—Two dogs were similarly injected with 5 and 10 cc of suspensions of *B. prodigiosus*. From one dog this organism was obtained from chyle removed 10 and 65 minutes after injection. Cultures of lymph from the other dog remained sterile.

Exper. 4.—Two dogs were fed daily for 15 days with 5 cc of a 24-hour dextrose bouillon suspension of *B. prodigiosus*. From one dog the organisms were not recovered in repeated cultures of the stool. From the second dog, *B. prodigiosus* was obtained from the stools on 4 separate occasions.

Exper. 5.—Five cc of a suspension of *B. prodigiosus* were introduced by stomach tube into 2 dogs, and under ether anesthesia the thoracic lymph cultured at 5 minute intervals for 2 hours; no organisms were recovered nor was *B. prodigiosus* found in specimens from the intestinal tract cultured 2½ hours after ingestion. In a third dog, 200 cc of a heavy suspension of *B. prodigiosus* were introduced by stomach tube, and cultures were made as before. No organisms were isolated from the chyle, though 1½ hours after ingestion this organism was found in the stomach, jejunum, and ileum.

¹⁴ Unpublished work.

Exper. 6.—Six dogs were each given 5 compound cathartic pills, and fairly active catharsis was obtained. Two hundred and fifty c c of *B. prodigiosus* were injected by stomach tube. Four hours later, the dog was killed, and cultures were made of lymph from the cisterna chyli, blood from the heart and portal vein, portions of liver and spleen and mesenteric lymph glands. *B. prodigiosus* was not recovered from these places, though it was present in the stomach, ileum and colon.

Exper. 7.—Three dogs were fed 5 compound cathartic pills daily for from 5 to 7 days, maintaining an active catharsis. Two hundred and fifty c c of a suspension of *B. prodigiosus* were introduced into the stomach by stomach tube, and the dogs were killed 4 hours later and cultures made as before. *B. prodigiosus* was not recovered from specimens of chyle, heart and portal blood, liver, spleen or mesenteric lymph glands, though this organism was isolated from the ileum and cecum.

SUMMARY

In our experiments with dogs using suspensions of *B. pyocyaneus*, *B. prodigiosus* and *Streptococcus hemolyticus* introduced by stomach tube, we were unable to recover the test organisms from the chyle, blood, or organs. When bacteria were introduced into the duodenum by needle puncture, in one of the 6 dogs, *B. pyocyaneus* was recovered from the thoracic duct 80 and 105 minutes after injection, and similarly *B. prodigiosus* was recovered 10 and 65 minutes after injection in one of two dogs. In these experiments, leakage from the puncture and resulting direct passage from the upper peritoneum by way of the lymphatics to the thoracic duct cannot be excluded. It should be noted, however, that the passage of organisms from the peritoneum to the thoracic duct is rapid, and such organisms usually reach the duct from this source in from 5 to 15 minutes. In one of our positive results, the organisms appeared only after the lapse of an hour. Ravenel¹⁵ encountered a somewhat similar difficulty in his experiments in which tubercle bacilli were injected by needle into the stomachs of young guinea-pigs. It is also possible that organisms may have entered the lymphatics directly through the minute lesion of the intestinal wall caused by the needle puncture itself. Under ether anesthesia, movements of the intestine are decreased, and presumably the lymph flow from the intestine thereby lessened, so that under these experimental conditions the opportunity for prompt transport and consequent detection of such organisms as pass the intestinal barrier may be less than under normal conditions. Feeding experiments performed after the production of active catharsis were also negative with the test organisms used.

There seems to be no question as to the passage of certain resistant organisms, such as tubercle and anthrax bacilli, through the intact

¹⁵ Jour. Med. Research, 1908, 18, p. 1.

intestinal mucosa. The less resistant organisms, such as those we have used, rarely pass the intestine of healthy animals. Under conditions in which resistance to infection is decreased or when unusually hardy organisms enter the bowel, or when lesions of the bowel are present, it is reasonable to suppose that bacteria may enter the blood stream from this source.

STUDIES IN COMPARATIVE IMMUNITY

I. RESISTANCE OF THE FROG TO STAPHYLOCOCCUS AUREUS

WITH ONE PLATE

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That bacteria and toxins virulent to one animal frequently are quite avirulent to another even closely related animal, would indicate that generalizing from the study of single factors will have to give place to a broader consideration of many factors (histologic, chemical, physical, etc.) operating in individual instances of immunity. The saying that "What is one man's food is another man's poison" is especially true of the action of bacteria and their products. But until we know more of the physicochemical conditions governing the growth of bacteria in vivo, our efforts must necessarily remain limited to the study of the grosser biologic manifestations of immunity. In the ultimate analysis of immunity there probably will be discovered factors that are specific only for closely related animals. With this idea in mind I decided to study the immunity of the frog to the staphylococcus. An attempt to identify as many factors as possible in the resistance of the frog to experimental infection with this organism, and a comparison of their relative effectiveness is the object of this paper.

The strain of *Staphylococcus aureus* used was recovered from the blood in acute endocarditis and multiple arthritis. Twenty-four hour plain agar cultures were washed off with salt solution, each c.c. of suspension containing the growth of 2 tubes, approximately 5 billions staphylococci. In all experiments the common laboratory frog (*Rana vernalis*) was used.

¹ NATURAL RESISTANCE OF FROGS TO STAPHYLOCOCCUS INFECTION

Expt. 1.—Eighteen frogs were injected in different lymph sacs, or muscles of thigh or tongue, each frog receiving 1 cc. of bacterial suspension. The frogs were examined 24 hours later and did not seem to be affected by the injection. By means of a fine pipet a few drops of fluid were withdrawn from the injected lymph sacs, and examination showed that most of the bacteria had disappeared, as a few free cocci and several polymorphonuclear leukocytes filled with gram-positive cocci were found in the fluid. Two, three, and four days after the injection the frogs were apparently well.

Expt. 2.—Three frogs were injected intraperitoneally with 1 cc. of staphylococcus suspension, and the next day all were ill. They were somewhat more

lively the following 2 days. Seventy-two hours after injection one of the frogs was killed. On opening the peritoneal cavity the viscera were found covered with a yellowish fibrinous exudate, which consisted of masses of polymorphonuclear leukocytes packed with staphylococci and held together by fibrin. A second frog was killed on the 5th day after injection, and a few small areas of fibrinous exudate were found in the peritoneal cavity. The third frog was killed on the 8th day after injection. No exudate was found.

Exper. 3.—Three frogs received injections of staphylococcus suspension into the anterior chamber of the eyes. The eyes were emptied of vitreous humor by means of a fine pipet, and refilled with the bacterial suspension. Twenty-four hours later 2 of the 6 injected eyes were swollen and cloudy (2 frogs). On the next day 1 frog seemed completely recovered; the other 2 were each blind on one eye; 4 days after the injection fluid withdrawn from one of the cloudy eyes contained many phagocytes filled with staphylococci, much fibrin, and a few free cocci. It yielded a pure culture of *Staphylococcus aureus*. Eight days after injection, the eyes were as cloudy as before, and when inoculated on plain agar the fluid yielded a pure culture of *Staphylococcus aureus*. One eye was removed for microscopic examination. The sections showed an exudate, consisting of numerous polymorphonuclear leukocytes containing large numbers of intracellular cocci and of much fibrin, filling the anterior chamber of the eye. The other eye yielded positive cultures as late as 15 days after injection. It gradually cleared up, and 4 weeks after injection looked normal, except for small scars at the punctures.

Repeated injections into various parts of the body of frogs of heavy suspensions of a strain of *Staphylococcus aureus* highly virulent to man failed to produce suppuration, the only reactions being in the peritoneum and the anterior chamber of the eye, and consisting of a marked local leukocytosis and fibrinous exudate. These experiments show that the frog is highly resistant to experimental infection with staphylococci.

RÔLE PLAYED BY LOW BODY TEMPERATURE IN NATURAL IMMUNITY OF THE FROG

Since Pasteur and Joubert¹ showed that the natural immunity of fowls to anthrax was lost when inoculated fowls were immersed to the thighs in cold water until their temperature had been reduced, it has been accepted by many that the high temperature of the fowl was the direct cause of such immunity, the temperature being too high for the growth of anthrax bacilli. This explanation is given in textbooks on immunity, despite the fact that, as Wagner² showed, the anthrax bacillus develops readily in vitro in the blood and blood serum of fowls at a temperature as high as 42 to 43. The same interpretation exists of the rôle of the low body temperature of the frog in resisting anthrax infection. Gibier³ found that frogs lose their natural resistance to anthrax and succumb when after injection they are kept at a temperature of about 37 C. On the basis of Gibier's report, statements are made that the natural resistance of frogs to anthrax is lost when frogs are kept at

¹ Bull. de l'Acad. de méd., 1878, 7, p. 440.

² Ann. de l'Inst. Pasteur, 1890, 4, p. 570.

³ Compt. rend. Acad. de sc., 94, p. 1605.

a temperature of 25-35 C. As a matter of fact, Mesnil⁴ found that the green frog (*Rana esculenta*), a species that readily adapts itself to 35 C., resists even then infection with anthrax; while the brown frog (*Rana temporaria*), which adapts itself only slightly to high temperatures, dies whether it has been injected or not, when placed at 35 C. Also as Sabrazes and Colombot⁵ found, the low body temperature of *Hippocampus* (14-16 C.) did not protect that small osseous fish against experimental anthrax, and Kovalevsky⁶ produced fatal anthrax in the house cricket at 22-23 C. Jordan⁷ states that toads are very susceptible to anthrax. These facts indicate that the rôle of the low body temperature per se in natural immunity is exaggerated.

I have tried to overcome the possible influence of the low body temperature of the frog in the following ways:

Exper. 4.—(a) Twelve frogs were kept in the incubator, the temperature of which was gradually raised. The strain of frogs in this work endured well a temperature of 32-34 C. while a temperature above 35 C. invariably killed all injected, as well as controls, within 48 hours. These "warm" frogs were injected into the dorsal lymph sacs with a heavy suspension of *Staphylococcus aureus*; 24 hours later all frogs were well. Smears from the fluid of the sacs showed that most of the cocci had disappeared; 48 and 72 hours later all frogs were well.

(b) *Staphylococcus aureus* was grown on plain agar at room temperature and then passed through 8 frogs kept at room temperature. Six frogs were then injected with heavy suspensions of the "cold" strain of staphylococci. Examined 24, 48 and 72 hours after injection, all were well.

It is evident from these experiments that in the resistance of the frog to staphylococcus infection the low body temperature is of little or no importance.

DISTRIBUTION AND FATE OF STAPHYLOCOCCUS AUREUS IN BODY FLUIDS AND ORGANS OF THE FROG

As we have seen, most of the staphylococci injected into the lymph sacs disappeared within 24 hours. In order to study the fate of the injected bacteria and the site of their destruction, the following experiments were made.

Exper. 5.—Thirty-two frogs were given injections of a heavy suspension of *Staphylococcus aureus* into the dorsal lymph sac, killed at given intervals, two at a time, and smears made from the heart blood, the fluid of the injected lymph sacs, the bile, and the bone marrow, and stained after Gram's method. Leukocyte counts were also made. The heart, lung, liver, spleen and kidney were removed for histologic examination. The results are given in table 1.

⁴ Ann. de l'Inst. Pasteur, 1895, 9, p. 301.

⁵ Ann. de l'Inst. Pasteur, 1894, 8, p. 696.

⁶ Bull. Acad. de sc. St. Petersburg, 1894, p. 437.

⁷ Text-Book of Bacteriology, 1916, p. 239.

Cultures were made from the heart blood and from the lymph of the injected sacs, and also from the liver, after grinding the tissue with sterile sand. The last positive culture from the blood was obtained on the 3d day after injection, from the lymph on the 5th day, and from the liver on the 6th day.

Counts showed that an increase in leukocytes began 2 hours after injection, reached a maximum of about 30,000 per cmm. in the 4th hour, and returned to normal on the 3d day.

A study of table 1 shows the following: Free staphylococci appeared in the blood stream 15 minutes after injection, reached a maximum in 30 minutes; a few free cocci were seen 3 hours after injection, but all had disappeared from the blood stream 6 hours after

TABLE 1
THE DISTRIBUTION OF STAPHYLOCOCCI IN THE BODY FLUIDS OF THE FROG

Time After Injection	Blood		Lymph		Bone Marrow		Bile
	Free Staphylo-cocci	Phago-cytes*	Free Staphylo-cocci	Phago-cytes	Free Staphylo-cocci	Phago-cytes	
15 minutes.....	† +	+++++++	0	0	0	0
30 minutes.....	+++++	+	+++++++	0	0	0	0
45 minutes.....	+++	+++	+++++	0	++	+	0
1 hour.....	+++	+++	+++++	++	+	++	0
2 hours.....	++	+++	+++	++	+	+++	0
3 hours.....	+	++	+++	+++	0	++	0
4 hours.....	+	++	+++	+++	0	+	0
6 hours.....	0	++	+++	+++	0	0	0
24 hours.....	0	+	+	++	0	0	0
48 hours.....	0	0	0	+	0	0	0
72 hours.....	0	0	0	0	0	0	0

* By phagocytes are meant polymorphonuclear leukocytes containing bacteria.
† +, few; ++, a moderate number; +++, many; +++++, very many; ++++++, "loaded" with.

injection. Only a few phagocytes were found in the blood 30 minutes after the injection; they became marked 45 minutes after the injection, remained conspicuous for 6 hours, and disappeared in about 48 hours. Though a positive blood culture was obtained 72 hours after the injection, on smears no bacteria were seen.

In the injected lymph sacs moderate phagocytosis began about 1 hour after injection, became marked in 3 hours, and then gradually disappeared; no bacteria were seen in smears 72 hours after injection, although the fluid yielded positive cultures for 48 hours longer.

The bone marrow played a modest part in the destruction of the injected bacteria, which apparently, reached the marrow by way of the blood stream; the bile remained bacteria free.

While phagocytosis in the blood and at the point of injection played an active rôle in the destruction of the injected bacteria, and while most of the phagocytes were packed with cocci almost to the bursting point, the fact is that not more than 1 of every 4 or 5 polymorphonuclear leukocytes seen contained cocci. Therefore, considering the large number of bacteria injected, not less than 5 billions, phagocytosis could not account for the destruction of all bacteria. Assuming a leukocytosis of 30,000 per c mm., and 1 of every 5 leukocytes a phagocyte, 2 c c. of blood found in the average frog which I have employed would contain $6,000 \times 1,000 \times 2$, about 12,000,000 phagocytes; at 100 cocci to each phagocyte the total number of staphylococci destroyed by the phagocytes of the blood would be about 1.2 billions, or between one-fourth and one-third of the number injected.

THE RÔLE OF THE BODY FLUIDS IN THE RESISTANCE OF THE FROG TO STAPHYLOCOCCUS AUREUS INFECTION

In vitro the blood serum of the frog proved a good culture medium for the staphylococci used, both at room and incubator temperature. A luxurious growth was also obtained when the coccus was planted on agar covered with a thick layer of fresh frog blood. Normal frog serum contained no agglutinins; nor did I observe agglutinins in the serum of a frog that had received 5 injections of staphylococcus suspension at 3 day intervals.

The fact that positive cultures were obtained from the lymph 5 days after injection and from the blood 3 days after injection would indicate that in vivo also the body fluids of the frog have little destructive power for *Staphylococcus aureus*.

Since only about one-fourth of the available phagocytes were found active in the experiments, the majority of the injected bacteria having been taken care of by some other agent, I tried to find out to what extent the phagocytes could be relied on in case the number of injected bacteria were so great as to tax all possible agents of defense. Therefore I injected 6 frogs with such bacterial suspension that each received about 25 billion bacteria; 24 hours later the injected lymph sacs were found distended with fluid, but the frogs did not appear ill. By means of a fine pipet some of the fluid was withdrawn. It was clear and contained a few flocculent particles. It coagulated rapidly, and did not differ from the normal contents of the lymph sacs except in quantity. Microscopic examination showed few free cocci and several phagocytes filled with cocci.

The distention of the sacs was less 3 days after injection, but there still were seen occasional free cocci in the fluid. One of the frogs was killed, and it was found that a thick layer of whitish fibrinous exudate covered the wall of the injected sac. The exudate was made up of a few free cocci and of numerous leukocytes filled with cocci closely packed and held together by a large mass of fibrin. The other 5 frogs were killed, 1 on each of the following 5 days. In the 4 and 5 day frogs the exudate was still abundant. There was less exudate in the 6 and 7 day frogs. In the last frog killed 8 days after injection, there remained some brownish, dry exudate, which gave a scant growth on agar, the pigment forming only 3 days after inoculation.

The presence of a fibrinous exudate in exper. 2 and 3 while absent in exper. 1, may be explained as follows: The lymph sacs communicate directly with the vascular system, and the injected bacteria readily pass into the blood stream in large numbers; therefore the local accumulation of leukocytes is small. In the abdominal cavity and in the eye the anatomic conditions make the passage of the injected bacteria far more difficult and hence delayed, and the masses of leukocytes that begin to accumulate at the point of inoculation in about an hour after the injection block the capillaries and prevent still farther the passage of the bacteria into the general circulation. Therefore, in these cases local phagocytosis and walling off of the injected bacteria by masses of leukocytes and fibrin are the chief means of destruction of the injected bacteria.

In exper. 4 the number of injected bacteria was extremely large. Therefore, even if many bacteria did pass into the general circulation, the accumulation of leukocytes at the point of inoculation in about an hour after the injection prevented the majority of the injected bacteria from passing into the blood stream, the accumulated masses of leukocytes blocking the capillaries. In this case the destruction of the bacteria was chiefly within the localized phagocytes.

In the last experiment certain facts were observed that deserve special attention. As we have seen, free staphylococci were present in the lymph sac 3 days after injection. The free staphylococci did not multiply. They should have multiplied and developed a strain of staphylococci resistant to the frog's leukocytes and therefore virulent to the frog, if the teaching of Metchnikoff and his followers in this respect is correct. There also was no evidence of struggle on the part of the bacteria, since the phagocytes showed no ill effects even when filled with staphylococci almost to the bursting point. Evidently

the staphylococci in the body of the frog produced little or no leukocidin, and behaved more like inert digestible foreign material than like living organisms.

In order to study the reaction of the frog to inert foreign material, I injected into the dorsal lymph sacs of frogs liquid petrolatum, olive oil, and a suspension of casein in salt solution. The frogs thus injected were killed between the 4th and 7th days after injection, and in each frog was found masses of leukocytes held together by fibrin encapsulating the unabsorbed fluid that had been injected. We find here that the local reaction to inert material was quite the same as to live staphylococci.

ACCUMULATION OF STAPHYLOCOCCI IN THE KUPFFER CELLS OF THE LIVER

Paraffin sections were made of the heart, lung, liver, spleen and kidney of the 32 frogs that were used in the study of the distribution and fate of staphylococci in the fluids of injected frogs, as detailed in exper. 5. The sections were stained by Gram's method and counter-stained with acid carmin. The detailed findings follow:

After 30 minutes: Only a few leukocytes containing intracellular cocci, and a few free cocci were found in the capillaries of the liver. No cocci were found in the other organs.

After 45 minutes: Several phagocytes and a few free cocci seen in the blood spaces in the wall of the heart; occasional clumps of cocci seen in the capillaries of the lungs; few free cocci and several phagocytes found in the small blood vessels of the spleen; considerable numbers of cocci seen in phagocytes in the capillaries, few in the larger vessels; no cocci found in the kidney.

After 1 hour: There were more free and intracellular cocci in the blood spaces of the heart wall than seen in the 45 minute frogs, but the total number was small. The lung and kidney sections contained only few cocci; considerably more cocci were present in the spleen than in the other organs, mostly in the capillaries and within phagocytes. Many cocci were present in the capillaries of the liver, mostly intracellular, and few in the larger vessels.

After 2 hours: The heart, lung and kidney sections showed only occasional bacteria, all intracellular. The spleen contained many intracellular cocci, all in the capillaries; the capillaries of the liver were loaded with cocci, almost all of them being within the endothelial cells lining the capillaries. Few intracellular and no free cocci found in the larger vessels.

After 3 hours: A few cocci found within phagocytes in the heart wall but none free. No bacteria in the lung and kidney sections. In the spleen the number of cocci was about the same as in the 2 hour frogs; many cocci within the endothelial cells of the capillaries of the liver (fig. 1). Only a few were seen in leukocytes in larger vessels.

After 4 hours: No bacteria found in sections of heart, lung or kidney. In the spleen and liver the number and distribution of bacteria were about the same as in the 3-hour frogs.

After 6 hours: The findings were about the same as in the 4-hour frogs.

After 24 hours: In the spleen a few cells containing cocci were seen: bacteria were numerous in the endothelial cells of the capillaries of the liver. None in the larger vessels.

After 2 days: Only occasional cocci in the spleen; many cocci found in the endothelial cells of the liver capillaries, though somewhat fewer than in the 24-hour frogs.

After 3 days: No bacteria found in the spleen. The number of bacteria in the liver was decreasing, but was still large. The bacteria were definitely confined to the stellate endothelial cells of the capillaries, these cells corresponding exactly to the Kupffer cells as seen in control sections from livers of frogs vitally stained with trypan blue (fig. 2). The Kupffer cells containing bacteria were swollen and projected into the lumen of the capillaries.

After 4 days: No bacteria in the spleen. The number of bacteria in individual Kupffer cells had decreased, nor were there as many Kupffer cells containing bacteria as in the previous frogs (fig. 3).

After 7 days: No bacteria in the spleen. The liver sections showed only a few Kupffer cells containing small numbers of cocci (fig. 4).

After 9 days: No bacteria found in the Kupffer cells of the liver.

From these results it is seen that the bacteria that escaped into the general circulation were practically all taken up by the Kupffer cells of the liver, where they were slowly digested, except for a relatively small number of bacteria that were destroyed in the spleen. The approximate relative numerical distribution of the injected bacteria in the organs of the frog at various intervals is given in table 2.

TABLE 2

DISTRIBUTION AND RELATIVE NUMBER OF STAPHYLOCOCCI IN THE ORGANS OF THE FROG

Time After Injection	Liver	Spleen	Heart	Lung	Kidney
30 minutes.....	+	0	0	0	0
45 minutes.....	++	+	+	+	0
1 hour.....	++++	+++	++	+	+
2 hours.....	++++++	+++	+	+	+
3 hours.....	++++++	+++	+	0	0
4 hours.....	++++++	+++	0	0	0
6 hours.....	++++++	+++	0	0	0
1 day.....	++++++	+	0	0	0
2 days.....	+++++	+	0	0	0
3 days.....	++++	0	0	0	0
4 days.....	+++	0	0	0	0
7 days.....	++	0	0	0	0
9 days.....	0	0	0	0	0

Comparing tables 1 and 2, we find that by the time free staphylococci have disappeared from the general circulation (about 3-4 hours after injection), the Kupffer cells of the liver reach the maximum of phagocytic activity (fig. 1). Apparently the cocci in the blood when passing through the capillaries of the liver are taken up by the Kupffer cells until the blood becomes bacteria free.

Metchnikoff considered the Kupffer cells of the liver as leukocytes that had become arrested while passing through the capillaries of the liver. He grouped them with the macrophages, and as such thought they were chiefly concerned with phagocytosis of elements other than bacteria. This opinion presumably led Metchnikoff to overlook completely the extent of the phagocytic activity of the Kupffer cells in frogs injected with anthrax bacilli, when citing⁸ the work of his pupil Mesnil⁴ on immunity of the frog to anthrax infection. Mesnil found that shortly after intravenous injection of anthrax bacilli into frogs large numbers of bacilli were found in the "pigment cells" and Kupffer cells of the liver, and concluded that the liver played an important part in the destruction of anthrax bacilli. Hess⁹ considered the phagocytic activity of the leukocytes as useful chiefly in bringing the anthrax bacilli to the Kupffer cells to be destroyed by them. Voswinkel¹⁰ transfused frogs with salt solution until all leukocytes were washed out. He then injected anthrax bacilli and found that few bacilli remained in the circulation 3 hours after injection, the majority of the injected bacilli having accumulated in the capillaries of the liver. Schilling¹¹ found that in rabbits injected intravenously with suspensions of streptococci and staphylococci the Kupffer cells of the liver showed marked phagocytic activity as early as 30 minutes after injection. Kyes¹² found certain specialized endothelial cells in the spleen and liver of pigeons which constantly contain red blood corpuscles. When pigeons were given intravenous injections of pneumococci, Kyes¹³ found that most of the injected bacteria were taken up by these special cells of the liver, and to a lesser extent by the spleen. He considers the mechanism of intracellular destruction of pneumococci by the fixed phagocytes as the chief factor in immunity of the pigeon to pneumococci. His findings were corroborated by Berry and Melick,¹⁴ who injected pigeons with pneumococci intraperitoneally.

As we have seen, in frogs injected with *Staphylococcus aureus* there are two definite and separate mechanisms for the destruction of the bacteria. Locally the leukocytes of the blood and lymph showed marked activity. The bacteria that reached the general circulation, however, were destroyed by the fixed phagocytes, i. e., the Kupffer cells of the liver. The effectiveness of each of the two protective mechanisms is equally marked, almost all injected bacteria having been destroyed by one or the other or both.

As seen in fig. 2, the Kupffer cells containing the vital stain (trypan blue) are found in close proximity to the pigment-containing cells of the liver capillaries normally present in the frog. In the livers of frogs injected with staphylococci, those pigment cells were frequently found in close relation to the phagocytic Kupffer cells filled with bacteria, but themselves almost never containing bacteria. We note here that while in the pigeon one Kupffer cell performs two functions,

⁸ Immunity, 1905.

⁹ Virch. Arch. f. path. Anat., 1887, 109, p. 365.

¹⁰ Fortschr. d. Med., 1900, 8, p. 9.

¹¹ Virch. Arch. f. path. Anat., 1909, 196, p. 125.

¹² Internat. Monatshr. f. Anat. & Phys., 1914, 31, p. 543.

¹³ Jour. Infect. Dis., 1916, 18, p. 125.

¹⁴ Jour. Immunol., 1916, 1, p. 47.

in the frog there are Kupffer cells especially concerned with pigment metabolism and other Kupffer cells concerned with the destruction of bacteria.

The brown pigment found in the pigment cells in the frog liver is iron free. I have used both the Perl's Prussian blue and the Turnbull blue methods in staining for iron, but always with negative results. Eppinger¹⁵ could find no iron in the Kupffer cells of the normal liver in man; but he found iron pigment in the Kupffer cells in icterus hemolyticus. He also found that in pernicious anemia in man the Kupffer cells frequently contained red blood cells. We note that a process which is physiologic in the pigeon is found in man apparently only in pathologic conditions.

We see in the Kupffer cells of the liver an active phagocytic apparatus, indeed under certain conditions far more active than the phagocytic leukocytes. An idea as to the, so to speak, numerical strength of the Kupffer cells may be obtained from the figures given by Nathan¹⁶ who counted about one Kupffer cell to every 13.6 liver cells. Judging from the photomicrographs of the frog liver, there may be more than that number in the frog. In the injected frogs the Kupffer cells were always more prominent, and their shape frequently altered, the inclusion of foreign material, such as bacteria or particles of dye, producing a bulging of the cells into the lumen of the capillaries. Whether the shape of the cells was altered as a result in part at least, of increased functional activity, and whether the number of Kupffer cells could be increased by stimulating them to further activity, I am not prepared to state with certainty. That proliferation and the formation of multinuclear Kupffer cells do occur under certain conditions (injection of tubercle bacilli into rabbits), has been reported by Evans, Bowman and Winternitz.¹⁷

Considering that each two cords of liver cells are bounded by blood capillaries whose endothelial lining is constantly exposed to various injurious agents that may circulate in the blood, it is not surprising that these cells possess the power to take up and destroy the injurious agents. Such an apparatus forming a syncytium of endothelial cells enveloping practically every cell of the liver should also form an efficient protective mechanism for the liver itself.

Since the Kupffer cells of the liver are energetically and effectively phagocytic in animals under certain conditions, why as Schilling found,

¹⁵ Enc. Klin. Med., Die Hepato-Lienalen Erkrank., 1920.

¹⁶ Jour. de L'Anat. et Physiol., 1908, 44, p. 208.

¹⁷ Jour. Exper. Med., 1914, 19, p. 283.

are bacteria so rarely found in the Kupffer cells of the human liver? Analyzing the experiments in which bacteria were found in the Kupffer cells, we find that phagocytosis was marked and effective in injected animals refractive to the organisms injected. Evans, Bowman and Winternitz found that the number of tubercle bacilli taken up by the Kupffer cells of rabbits was small (3-4 bacilli to a cell), and in animals killed 36 hours after injection the phagocytic Kupffer cells were found to have formed multinuclear giant cells containing one or more bacilli—a typical miliary tubercle. Here we encounter the same phenomenon that exists in connection with phagocytosis by leukocytes: when a given organism is virulent, it is not readily taken up; and when phagocytosis in such cases does occur, it is neither pronounced nor effective, the phagocytic cells eventually perishing. Is it not probable that the absence of bacteria in the Kupffer cells of the human liver is due to the fact that the bacteria that caused death were too toxic or too virulent for Kupffer cell phagocytosis? The study of the effects of virulent bacteria and toxins on the Kupffer cells of various animals and of the rôle of Kupffer cells in antibody production is now under way and will be reported later.

SUMMARY

It is shown that the common laboratory frog (*Rana vernalis*) is highly refractive to a strain of *Staphylococcus aureus* virulent to man. This immunity does not depend on the low body temperature of the frog.

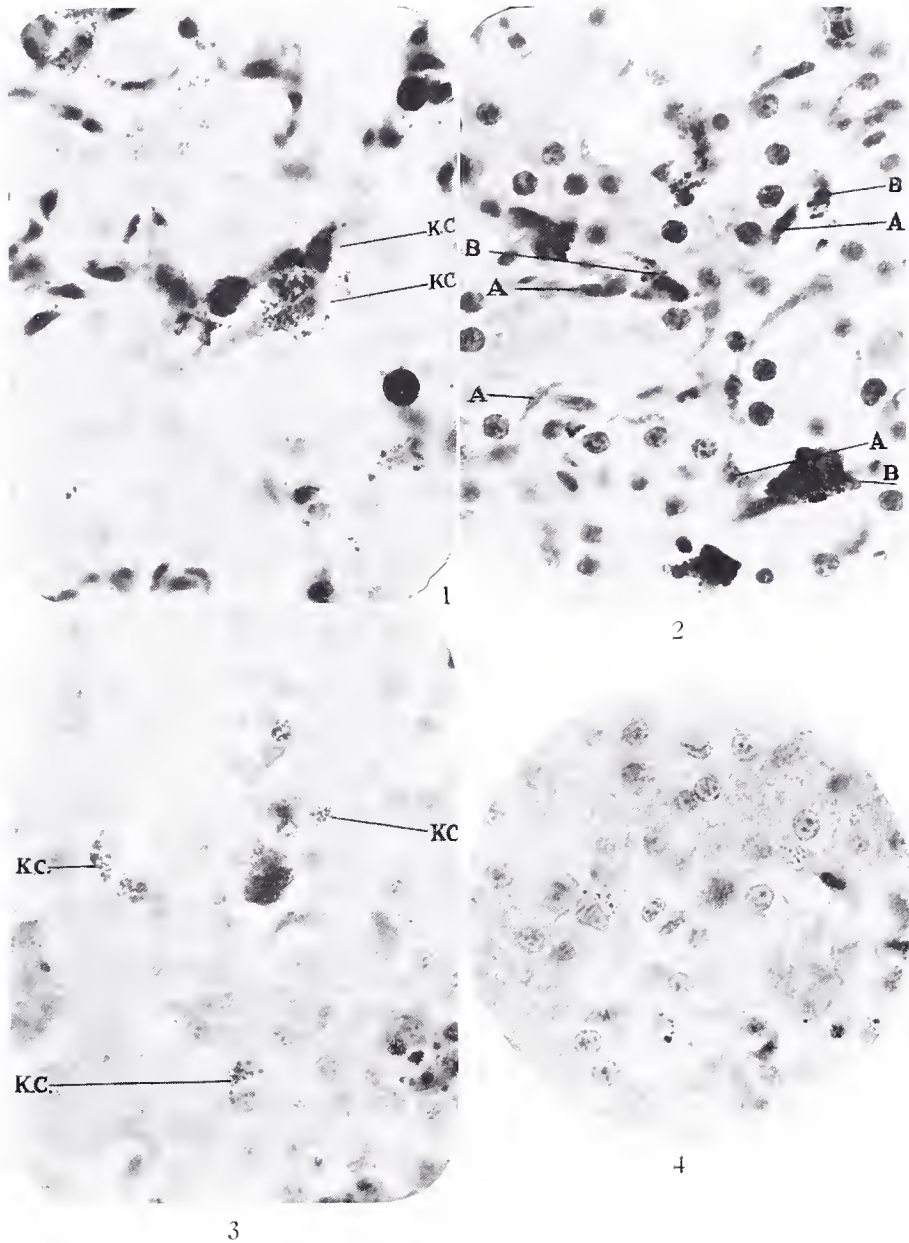
Positive cultures were obtained from the heart blood of frogs 3 days after injection, from the lymph 5 days, and from the vitreous humor of the eye as late as 15 days after injection, showing that the body fluids of the frog are not highly bactericidal to *Staphylococcus aureus* in vivo.

There are present in the frog two well defined mechanisms for the destruction of staphylococci. When the injected bacteria pass readily into the general circulation, the Kupffer cells of the liver show marked phagocytic activity, and in a few hours almost all bacteria that were circulating in the blood stream are found within the Kupffer cells where they are slowly digested.

When the anatomic conditions prevent the early passage of the injected bacteria into the general circulation, the phagocytic activity of the leukocytes form an effective local mechanism of defense.

While some injected staphylococci may remain alive in the frog for several days, they are, for some unknown reason, unable to exercise their vital activities in the living frog.

PLATE 1



EXPLANATION OF PLATE

Fig. 1.—Liver of frog 3 hours after injection of staphylococci into the dorsal lymph sac. *K C*, Kupffer cells packed with gram-positive cocci; $\times 650$.

Fig. 2.—Liver of frog vitally stained with trypan blue. *A*, Kupffer cells containing the vital stain; *B*, endothelial cells of liver capillaries normally containing brown pigment; $\times 650$.

Fig. 3.—Liver of frog 4 days after injection of staphylococci into the dorsal lymph sac. *K C*, staphylococci in the Kupffer cells; $\times 650$.

Fig. 4.—Liver of frog 7 days after injection of staphylococci into the dorsal lymph sac. The Kupffer cells still contain cocci; $\times 700$.

GROWTH OF PFEIFFER BACILLUS IN MIXED CULTURE IN BLOOD-FREE MEDIUM

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Attempts to grow the Pfeiffer bacillus in a blood-free medium have been made by a number of investigators. The results are quite conflicting. In 1901, Cantani¹ reported having grown this micro-organism on plain agar in mixed culture with the gonococcus and with *B. diphtheriae*. Ghon and von Preyss² shortly afterward repeated Cantani's work with negative results. Neisser³ claimed to have cultivated the Pfeiffer bacillus on plain agar in mixed culture with *B. xerosis* for 20 successive generations. Luerksen,⁴ in attempting to repeat Cantani's work, was unable to confirm his results with living organisms, but obtained abundant growth of the Pfeiffer bacillus on blood-free mediums in the presence of heat killed cultures of staphylococcus, *B. prodigiosus*, and *B. diphtheriae*.

More recently, Olsen,⁵ who tested various derivatives of hemoglobin for growth-promoting properties, found that hematin and hemin did not permit growth except close around stimulating colonies of other species of bacteria; and that hematoporphyrin did not permit growth even in the presence of stimulating organisms. Putnam and Gay⁶ failed to grow any one of 16 strains on blood-free medium in successive generations. They employed a number of alleged growth-promoting organisms in their experiments and a variety of mediums, but observed no growth-stimulating effect. Williams and Povitsky⁷ obtained positive results in a similar experiment. To eliminate the possible presence of minute quantities of hemoglobin, they used a blood-free wheat agar which contained no meat extract, but did contain peptone. On this medium, as well as on plain meat-infusion agar and dextrose-meat-infusion agar, they obtained growth of the Pfeiffer bacillus in mixed culture, both with *B. diphtheriae* and with the gonococcus through 22 successive generations. With a hemolytic streptococcus or a yeastlike organism as growth-promoting agents their results were less favorable. With a strain of *Staphylococcus aureus*, the Pfeiffer bacillus disappeared after a very few generations. This was also observed on heated blood agar ("chocolate agar"), which was used as control medium. Davis⁸ likewise noticed that the Pfeiffer bacillus is readily overgrown by the staphylococcus both on solid and in liquid blood mediums.

In view of these observations it seems of interest that I have cultivated the Pfeiffer bacillus for 52 successive generations in mixed culture

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¹ Ztschr. f. Hyg. u. Infektionskrankh., 1901, 36, p. 29.

² Centralbl. f. Bakteriol., 1, O., 1902, 32, p. 90.

³ Deutsch. med. Wchnschr., 1903, 29, p. 462.

⁴ Centralbl. f. Bakteriol., 1, O., 1904, 35, p. 434.

⁵ Ibid., 1920, 85, p. 12.

⁶ Jour. Med. Res., 1920, 42, p. 1.

⁷ Ibid., 1921, 42, p. 383.

⁸ Jour. Infect. Dis., 1921, 29, p. 178.

with staphylococcus in a blood-free medium made of 2% Armour's peptone and 0.5% sodium chloride in tapwater. The Pfeiffer bacillus does not grow alone in this medium.

Strains of *Staphylococcus albus*, *Streptococcus viridans* and *B. subtilis* (all nomindol producers) were tested as growth-promoting agents. Tubes with 6 c.c. amounts of blood-free peptone salt solution were inoculated with approximately equal quantities of 24-hour cultures of one of these bacteria together with an indol-forming, typical strain of the Pfeiffer bacillus. After 48 hours' incubation at 37 C., two or three large loops of these mixed cultures were transferred to fresh tubes of the same medium, and the 48-hour cultures were then tested for indol by adding 1 c.c. of Ehrlich's reagent. Forty-eight hours later the procedure was repeated. Corresponding cultures in a control medium of peptone salt solution, enriched with 2% of red cells of horse blood, and heated at 80 C. for 20 minutes, were carried during the entire experiment.

Jordan⁹ has suggested that the indol production by Pfeiffer bacilli may be usable under certain conditions as a "presumptive test" of the presence of these organisms. In my experiment, the Pfeiffer bacillus was assumed to be present in all indol-positive cultures. The mixed cultures were plated out on blood agar and oleate agar every fifth generation of the series to confirm this assumption. In all cases, when the indol reaction was positive, Pfeiffer bacilli were isolated. The experiment was conducted in this manner during 52 successive generations, covering a period of more than five months.

In the first test-tube inoculation of the blood-free mixed culture of Pfeiffer bacilli and streptococci, indol was not found, nor could a single colony of either of these organisms be found on the first plating of the corresponding culture. The strain of streptococcus used did not seem to grow in blood-free peptone salt solution, and its consequent absence as a growth-promoting agent probably accounts for the absence of growth of the Pfeiffer bacillus. The corresponding control tube of blood peptone salt solution was indol-positive. Colonies of both organisms were found on the first plating from this medium (5th test tube generation), but the second plating (after the 10th generation) showed Pfeiffer colonies only, indicating that the streptococci had been overgrown by the Pfeiffer bacilli.

⁹ Jour. Am. Med. Assn., 1919, 72, p. 1542.

In a mixed culture with *B. subtilis* the Pfeiffer bacillus was overgrown after the 12th generation in the control tube of blood-containing medium, but in the blood-free medium it was carried in 25 successive generations, after which time this culture ceased to show indol. Plates made at this time also were negative for Pfeiffer bacilli.

With staphylococcus as a growth-promoting agent, the Pfeiffer bacillus was carried for 52 generations, and it is still living in this mixed culture, even though a period of 25 days elapsed between the 41st and the 42nd, and one of 31 days between the 48th and the 49th generations. Both organisms were detected on oleate plates after the 50th generation.

Attempts to repeat this experiment using a medium free from animal constituents, have failed. As the facility with which the experiment can be conducted is dependent on the indol test, it was thought desirable to obtain a vegetable medium containing tryptophan. Wheat, rye, beans, peas, and hemp are known to contain tryptophan.¹⁰ Extracts from these vegetables were made as follows:

1. Soak 100 gm. of vegetable in 300 c.c. tapwater for 18 hours.
2. Autoclave 30 min. at 15 lbs.
3. Strain through cheese cloth.
4. Adjust reaction to P_H 7.5.
5. Tube and sterilize in autoclave.

These extracts, enriched with 2% red cells of horse blood, gave good growth of the Pfeiffer bacillus in 24 hours, but the growth could not be detected by means of the indol test (24, 48, and 72 hours) as no indol had formed. Testing the mediums with *B. coli* also gave a negative indol reaction. The bean and hemp extracts occasionally did give a faint positive reaction, both with the Pfeiffer bacillus and *B. coli*, but this was not constant and could not be relied on. Many attempts were made to grow the Pfeiffer bacillus in mixed culture with staphylococcus in blood-free bean and hemp extracts, but in no instance was growth observed in more than 3 successive generations. Parallel tests for growth were made on oleate plates and by transferring 48-hour cultures to peptone salt solution which was tested for indol after 48 hours' incubation. Pfeiffer colonies were always scarce on the plates, and not infrequently no Pfeiffer colonies could be detected, although the corresponding indol test in peptone salt solution was positive. A

¹⁰ Mathews, A. P.: Physiological Chemistry, Ed. 3, p. 129.

decreasing intensity of the indol reaction in successive generations suggested a gradual dying out of the Pfeiffer bacilli.

Fildes' potato extract¹¹ has been tested also. Both plain extract and extract enriched with 2% red cells of horse blood were used. Several tests for growth of the Pfeiffer bacillus were made, in pure culture as well as in mixed culture with staphylococcus, and all proved negative by both the oleate plate method and the indol method described above. *B. coli* did not form indol in this medium in 24 hours, but a slight positive reaction appeared in 48 hours.

It would seem that none of the vegetable extracts used are suitable for the experiment, first, on account of their inadequacy for sustaining the growth of the Pfeiffer bacillus, and secondly, because the amount of tryptophan contained is too small for sufficient production of indol for a satisfactory test. It should be noted that none of the workers referred to have reported growth in pure vegetable extracts. The wheat medium employed by Williams and Povitsky contained 1% peptone, and Fildes' potato extract was not used without addition of "bacto-peptone water."

SUMMARY

A typical, indol-forming strain of the Pfeiffer bacillus has been carried through a considerable number of successive generations in mixed culture in a blood-free medium with staphylococcus and *B. subtilis*.

The growth accessory substances may, therefore, be found outside of blood.

The "Pfeiffer presumptive test" has proved of practical application in these observations.

Vegetable extracts alone were not suitable for growth of the Pfeiffer bacillus, either in pure culture or in mixed culture with staphylococcus.

¹¹ Brit. Jour. Exper. Path., 1922, 3, p. 210.

IMMUNOLOGIC SIGNIFICANCE OF VITAMINS

I. INFLUENCE OF THE LACK OF VITAMINS ON THE PRODUCTION OF SPECIFIC AGGLUTININS, PRECIPITINS, HEMOLYSINS AND BACTERIOLYSINS IN THE RAT, RABBIT AND PIGEON

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The influence of vitamins on the production of antibodies has received little study. Guerrini¹ came to the conclusion that pigeons fed on a diet of polished rice lose the ability to elaborate agglutinins. Kleinschmidt² reported that hemolysin formation in the dog was subnormal when the animals were fed unsuitable diets, although Hektoen³ found the antibody production normal in rats fed on the Osborne-Mendel stunting food. Zilva⁴ found no disturbance in the production of agglutinins or hemolysins in the rat fed on a diet free of vitamin A or B; or in the guinea-pig on a ration low in vitamin C.

The purpose of this work was to determine the effect of a deficiency of vitamins A and B on the production of agglutinins, precipitins, hemolysins and bacteriolysins in the rat, rabbit and pigeon. The rat and rabbit were chosen because they are susceptible to a deficiency of vitamin A and the pigeon because it is susceptible to the lack of vitamin B. The rat also serves adequately in the study of vitamin B.

Numerous investigators^{1,5} have reported a break in the natural resistance to bacterial infection of animals suffering from the lack of vitamins. Apparently this cataphylaxis occurs more especially in animals which have received a ration deficient in vitamin A. The occurrence of xerophthalmia and subsequent infection in the rat and rabbit indicate a biologic derangement of the normal resisting powers of vitamin starved animals. This increase in susceptibility may be more mechanical than a derangement of the antibody forming processes as the result of an insufficient supply of vitamins.

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¹ Ann. d'iq., 1921, 31, p. 596.

² Monatschr. f. Kinderheilk., 1913, 12, p. 423.

³ Jour. Infect. Dis., 1914, 14, p. 279.

⁴ Biochem. Jour., 1919, 13, p. 172.

⁵ Rénon: Bull. gén. de therap., 1914, 168, p. 91; Muthu, C.: Brit. Med. Jour., 1920, 2, p. 160; Dutton, A. S.: Med. Press, 1920, 109, p. 313; Fleming, Macaulay and Clark: Report on the Prevalence and Prevention of Scurvy and Pneumonia in Southern Rhodesia Among Native Laborers.

METHODS

The following ration was employed to produce symptoms in the rat from the lack of vitamin A:

RATION 1

Casein (alcohol extracted).....	18%
Dextrin	74%
Salt mixture	5%
Yeast	3%

One month old rats when placed on this diet developed rough coats, lost weight rapidly, showed xerophthalmia, became sluggish and finally died unless vitamin A was supplied them. The same basic ration was used in diet 2 for the studies on vitamin B, except that butterfat was substituted for the yeast. The salt mixture was that recommended by McCollum and Davis.⁶ The following ration was fed to produce xerophthalmia in the rabbit (Nelson, Lamb and Heller):⁷

RATION 3

White corn	65%
Linseed oil meal.....	3%
Ground oats	22%
Casein (alcohol extracted).....	5%
Tankage	3%
Calcium carbonate	1%
Sodium chloride	1%

Young rabbits weighing 2,000 gm. were selected; xerophthalmia usually appeared after two months and was accompanied by great loss in weight. Animals suffering from the lack of vitamin A will be referred to in later discussions as test A animals, and those suffering from the lack of vitamin B as test B animals. Control animals were fed on the same ration that the test animals received with the addition of the missing vitamin, in butterfat in the case of vitamin A and in yeast in the case of vitamin B. Distilled water was supplied to all animals.

Polyneuritis was produced in the pigeon both by a diet of polished rice and by the use of ration 2. Forced feeding was required somewhat earlier with the polished rice than when ration 2 was fed, although there appeared to be no difference in the time required to produce the symptoms of the disease.

Bacterium typhosum was selected as antigen in the production of agglutinins in the rat, rabbit and pigeon. Human serum was employed as antigen in the precipitin tests in the rat, and *Bacterium typhosum* in the production of bacteriolysins in the rat and rabbit. Rabbit erythrocytes were used in the production of hemolysins in the rat and rat erythrocytes served as antigen in the rabbit.

The rat serum employed for agglutination, hemolysis and precipitation was obtained by bleeding from the tail; a partial vacuum was employed to insure a sufficient quantity. Rabbits were bled from the marginal vein of the ear and pigeons from a wing vein. Sterile blood was obtained for the bacteriolytic tests by heart puncture. No anesthetic was given in the case of either rats or rabbits.

⁶ Jour. Biol. Chem., 1915, 20, p. 649

⁷ Jour. Physiol., 1922, 59, p. 335.

The Dreyer technic⁸ somewhat modified was employed in the agglutination tests. A heavy suspension of formalized antigen was prepared and allowed to stabilize for several weeks, when a series of dilutions were made and tests made to determine the optimum concentration of antigen. These tests indicated that a dilution of one part antigen to one part salt solution produced the best agglutinations. This diluted antigen was used in all agglutination tests as standard antigen. Readings were recorded after one hour at 37 C.

A 5% suspension of corpuscles and guinea-pig complement were regarded as standard in hemolysin titrations; 0.5 cc. of inactivated hemolytic amboceptor was added in dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1,280. Controls were set up of 0.5 cc. corpuscle suspension plus 0.5 cc. complement and 0.5 cc. corpuscle suspension plus 0.5 cc. amboceptor (1:10), and both tubes made up to volume with salt solution. Readings were recorded after one hour at 37 C. A determination of the precipitin production was made on rats suffering from the lack of vitamin A injected with human serum. Readings were recorded after one hour at 37 C.

The plate culture method (Stern-Körte) was used in the bacteriolytic tests. Colony counts were made after 24 hours.

PRODUCTION OF AGGLUTININS IN THE RABBIT LACKING VITAMIN A

To determine the production of agglutinins in rabbits suffering from the lack of vitamin A, 11 animals were selected. Six of these had received a ration deficient in vitamin A for 12 weeks. Each rabbit was given intravenous injections of 0.2, 0.3, 0.4, 0.6, and 1.0 cc., respectively, of a killed 18-hour broth culture of *Bacterium typhosum* at intervals of 7 days. Xerophthalmia was distinctly present in 2 of the animals. The animals had lost weight after the first month on the diet and had acquired a rough thin fur. Subsequent to the second injection bleedings were made from each animal on the seventh day after inoculation. Representative results from 4 animals appear in table 1. The data show that agglutinin production in the animals lacking vitamin A proceeded as in the controls. The individual titers reached at the last bleeding were quite uniform, although slight fluctuations occurred. As these fluctuations were as great in the control rabbits as in the animals lacking vitamin A, they have no significance. A titer of 81,920 was reached by 1 animal, a test rabbit, while 2 others reached a titer of 40,960. Among the controls, 2 reached a titer of 40,960. The lowest titer, 10,240, was reached by both a test and a control rabbit.

The titer obtained in this experiment was quite high undoubtedly owing to the fact that the strain of *Bacterium typhosum* used was known to agglutinate strongly with immune serums. These results were substantiated by 3 other series of experiments.

PRODUCTION OF AGGLUTININS IN THE RAT LACKING VITAMIN A

To determine the reaction of the rat to a deficiency of vitamin A, 11 rats in advanced stages of avitaminosis were selected, together with 6 controls. Four injections at 7-day intervals of 0.2, 0.3, 0.6, and 1.0 cc., respectively, of an 18-hour broth culture of *Bacterium typhosum* were given into the tail of each rat. Five of the test rats died as a result of the dietary deficiency before the injections were completed. Each rat was bled from the tail on the seventh day after injection. Agglutination results were obtained for each succeeding

⁸ Med. Res. Council, Bull. No. 51.

week. The results obtained with rats agreed with those in the rabbit. The deficiency of vitamin A exerted no influence on the ability of the animals to produce agglutinins. Individual fluctuations were somewhat greater in the rat, but as the rat is an irregular producer of antibodies, the differences are not indicative. Three of the 6 test rats and 2 of the controls attained a maximum titer of 1,280. These results were substantially obtained in 6 series of experiments.

PRODUCTION OF AGGLUTININS IN THE RAT LACKING VITAMIN B

Extremely emaciated white rats that had received no vitamin B in their ration for 13 weeks were selected to determine the influence of vitamin B on agglutinin formation. Twelve rats comprised the original group of test animals. Five died on feed and 2 died after the first injection. Five normal

TABLE 1
AGGLUTININ PRODUCTION IN THE RABBIT LACKING VITAMIN A

Rabbit No.	Treatment	Weight (On Diet) 8/12 Gm.	Weight (First Injection) 11/4 Gm.	Agglutination Results												
				Date of Test	1:15	1:30	1:60	1:120	1:240	1:480	1:960	1:1,920	1:3,840	1:7,680	1:15,360	1:30,720
1	Test A	2,255	2,165	11/18	2	1	1	0	0	0	0	0	0	0	0	0
				11/25	4	4	4	3	3	2	1	0	0	0	0	0
				12/ 2	4	4	4	4	3	2	1	1	1	0	0	0
				12/ 9	4	4	4	4	4	4	4	4	3	2	1	0
2	Test A	2,325	2,210	11/18	4	4	3	2	1	0	0	0	0	0	0	0
				11/25	4	4	4	3	3	2	1	0	0	0	0	0
				12/ 2	4	4	4	4	3	3	2	0	0	0	0	0
				12/ 9	4	4	4	4	3	2	2	1	1	0	0	0
3	Control	2,310	2,730	11/18	3	1	1	0	0	0	0	0	0	0	0	0
				11/25	4	4	4	3	3	1	0	0	0	0	0	0
				12/ 2	4	4	4	4	4	4	3	2	1	0	0	0
				12/ 9	4	4	4	4	4	4	4	4	3	2	1	0
4	Control	2,140	2,650	11/18	3	3	2	1	0	0	0	0	0	0	0	0
				11/25	4	4	4	4	3	2	1	0	0	0	0	0
				12/ 2	4	4	4	4	3	2	1	0	0	0	0	0
				12/ 9	4	4	4	4	4	4	4	3	2	1	0	0

4 = agglutination abundant; 3, 2, 1 = decreasing agglutination; 0 = no agglutination.

rats were used as controls. All rats received 5 tail injections of 0.3 cc. of an 18-hour broth culture of *Bacterium typhosum* at 7-day intervals. Subsequent to the second injection, each rat was bled from the tail on the seventh day after injection. The results showed no significant difference in the production of agglutinins in test and control animals. Titers of 30,720 were attained by both test and control animals 7 days after the last injection. The individual fluctuations were negligible in both the test and control serums. Similar results were obtained in 6 additional experiments.

PRODUCTION OF AGGLUTININS IN THE PIGEON LACKING VITAMIN B

As birds may differ from mammals in their response to injections of antigen, pigeons were selected and fed a diet of polished rice. Considerable difficulty was encountered in measuring the agglutinin formation. Birds that showed symptoms of polyneuritis died in a few days, and there was no opportunity to continue inoculations. When an attempt was made to prolong

the lives of the birds by administration of small quantities of yeast, they revived only to die when the yeast was withdrawn. Several series of birds were started before satisfactory results were obtained. Obviously, the period of injection was considerably shortened when compared with rats or rabbits.

Thirteen pigeons were started on the polished rice diet. A general drop in body temperature was observed on the 19th day. Each pigeon in the test group and 3 controls received an intraperitoneal injection of 0.1 cc. of a killed 18-hour broth culture of *Bacterium typhosum*; on the 26th day each received 0.3 cc., and 0.5 cc. on the 33d day. Prior to injections the pigeons were bled from a wing vein and agglutinins determined. Before the date of first bleeding, 10 of the 13 pigeons had died, leaving 3 birds; 1 of these died while the others were being bled, and heart blood was obtained postmortem. Bird No. 2

TABLE 2
PRODUCTION OF AGGLUTININS IN POLYNEURITIC PIGEONS

Pigeon No.	Pigeon Treatment	Rectal Temperature, F., 9/10	Weight 8/22, Gm.	Agglutination Tests									Remarks
				Date of Test	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
1	Test Polyneuritic	99.8	175.0	9/17	2	1	tr	0	0	0	0	0	Died 9/17
2	Test Polyneuritic	99.6	170.0	9/17 9/24	2 4	1 3	0 2	0 1	0 0	0 0	0 0	0 0	Died 9/24
3	Test Polyneuritic	105.7	165.0	9/17 9/24 10/ 2	2 4 4	1 4 4	tr 3 4	0 2 2	0 1 2	0 1 1	0 0 0	0 0 0	Died 10/6
4	Control	107.8	170.0	9/17 9/24 10/ 2	3 4 4	2 4 4	1 3 4	0 3 3	0 2 3	0 1 2	0 0 1	0 0 0	
5	Control	109.6	180.0	9/17 9/24 10/ 2	3 4 4	1 3 4	1 2 3	0 1 2	0 0 0	0 0 0	0 0 0	0 0 0	
6	Control	109.9	185.0	9/17 9/24 10/ 2	2 3 4	1 2 4	1 2 3	0 1 2	0 0 1	0 0 0	0 0 0	0 0 0	

4 = agglutination abundant; 3, 2, 1 = decreasing agglutination; tr = trace; 0 = no agglutination.

was moribund when bled for the second series of determinations and died immediately after. In spite of these extreme conditions of the test, agglutinins were produced as vigorously in polyneuritic birds as in normal healthy control birds (table 2). In all attempts to determine the production of agglutinins, the polyneuritic pigeons showed agglutinin formation comparable to controls.

It is to be noted that Guerrini¹ reported the inability of polyneuritic pigeons to form antibodies (agglutinins).

Similar experiments were carried out with pigeons fed on the rat ration No. 2 containing no vitamin B. These birds developed polyneuritis as readily as those fed polished rice and required considerably less hand-feeding as they ate the diet almost until the appearance of symptoms, whereas birds on a diet of polished rice required forced feeding during the second week on the

diet. Injections of 0.1, 0.3 and 0.5 cc. of a killed 18-hour broth culture of *Bacterium typhosum* were given intraperitoneally at 7-day intervals to 7 test B and 3 control birds. Four of the test birds died before the first agglutination test. The 3 remaining pigeons tested weekly produced agglutinins in no observable manner different from the controls. The production was regular and reached substantially the same titer as the control birds. The results were practically the same as obtained in the case of rice fed pigeons.

PRODUCTION OF PRECIPITINS IN THE RAT LACKING VITAMIN A

The precipitin production would be expected to be closely related to the production of agglutinins. We know of no reason, on the other hand, why this must necessarily be true. Precipitins were, therefore, determined in 4 rats distinctly suffering from the lack of vitamin A and 4 controls. Each animal was given five 1.0 cc. intraperitoneal injections of human serum at 5-day intervals and bled on the seventh day after the last injection. A titer of 5,000 was recorded for one test and one control rat, while all animals attained a titer of not less than 1,000. The results exhibited no significant differences.

PRODUCTION OF HEMOLYSINS IN THE RABBIT LACKING VITAMIN A

Kleinschmidt² reported the subnormal production of hemolysins in dogs fed unsuitable diets. To determine the effect produced by the lack of vitamin A, 2 young rabbits were selected that had received a ration low in the vitamin for 9 weeks. These 2 and 2 control rabbits received 4 intravenous injections of 4.0 cc. of a 50% suspension of washed rat erythrocytes at intervals of 5 days. The animals were bled on the 7th day after the last injection and the hemolysin titrated. The 2 controls and 1 test animal reached the same titer (1:320), while the remaining test rabbit showed a titer of 1:640. These results were substantiated in other experiments.

PRODUCTION OF HEMOLYSINS IN THE RAT LACKING VITAMIN A

To make the conclusions more general that animals lacking vitamin A produce hemolysins equal to those produced by control animals, the hemolysin formation in the rat was determined in a manner similar to that in the rabbit. Six rats suffering from the lack of vitamin A and 6 control rats were given 6 tail injections of 0.5 cc. of a 50% suspension of washed rabbit erythrocytes at intervals of 5 days. Bleeding occurred 7 days after the last injection and the serums from test rats 1, 2 and 3 were pooled (group 1) and serums from test rats 4, 5 and 6 were pooled and placed in group 2. Controls were similarly pooled (groups 3 and 4). The hemolysin formation in the 2 test groups and 1 control group were substantially the same (1:160). Group 3 of the control animals showed a slightly increased titer (1:320), but when the irregularity of the rat as an antibody producer is considered the difference may be ascribed to individual fluctuations. These results were confirmed by later experiments.

PRODUCTION OF BACTERIOLYSINS IN THE RAT LACKING VITAMIN A

The bacteriolytic properties of the body fluids undoubtedly play a part in the resistance offered to infection. Table 3 refers to an experiment on the production of bacteriolysins in the rat fed on a diet deficient in vitamin A.

Fourteen 6 weeks' old rats were selected and placed on Diet 1, and after 6 weeks on this diet received 6 intraperitoneal injections of 0.2 cc. of a killed

18-hour broth culture of *Bacterium typhosum* at intervals of 5 days. Four controls received the same treatment. Seven test rats remained to be bled 7 days after the last injection. The test and control animals were bled from the heart and their serums pooled into "test" and "control" groups. The serums were inactivated at 56 C. for 30 minutes and the production of bacteriolysins determined. The results indicate that a break in resistance does not occur through the failure of the organism to produce bacteriolysins. Results were obtained in several experiments to substantiate the production of bacteriolysins in rats lacking vitamin A.

PRODUCTION OF BACTERIOLYSINS IN THE RABBIT LACKING VITAMIN A

Two young Belgian hares that had lost 225 gm. and 195 gm., respectively, on the diet deficient in vitamin A were given 6 intravenous injections at 5-day intervals of 0.3 cc. of an 18-hour killed broth culture of *Bacterium*

TABLE 3
PRODUCTION OF BACTERIOLYSINS IN THE RAT LACKING VITAMIN A

Rats	Immune Inactivated Serum in 1 C c. Volume, C c.	Guinea-Pig Complement (1-10)	<i>Bacterium</i> <i>typhosum</i> 1:500 Dilution 18-Hour Cul- ture, C c.	Bacteria per Loopful 40 C.			
				0 Min.	10 Min.	30 Min.	60 Min.
Test A 7 rats	1.0	0.5	0.1	245 310	0 0	0 0	0 0
	0.05	0.5	0.1	348 269	0 0	0 0	0 0
Control 4 rats	1.0	0.5	0.1	286 354	0 0	0 0	0 0
	0.05	0.5	0.1	347 295	0 0	0 0	0 3
Normal serum 3 rats	1.0	0.5	0.1	268 319	0 0	0 0	0 0
	0.05	0.5	0.1	198 267	167 211	133 143	157 119

typhosum. Two control rabbits received the same treatment. They were bled on the 7th day after the last injection. The serums were inactivated and the production of bacteriolysins determined. The results obtained were substantially the same as were obtained in the rat. Both test and control animals produced bacteriolysins.

PRODUCTION OF BACTERIOLYSINS IN THE RAT LACKING VITAMIN B

Although the 2 vitamin deficiencies have certain symptoms of starvation in common, the conclusion cannot be drawn that a lack of vitamin B does not influence the production of bacteriolysins from the results noted when vitamin A is withheld. A deficiency of vitamin B leads in an orderly manner to a series of symptoms which are quite constant and recognized. The lack of vitamin A, on the other hand, may not become evident for a considerable time, when the animal appears to be in great distress and soon dies. The onset of symptoms is often quite explosive. Xerophthalmia may or may not develop. For this reason the production of bacteriolysins in the rat lacking

vitamin B was determined. Six rats that had received a diet lacking vitamin B for 12 weeks together with 6 control rats were given 6 intraperitoneal injections of 0.2 cc. of an 18-hour killed culture of *Bacterium typhosum* at intervals of 6 days. Seven days after the last injection the animals were bled from the heart and the serums pooled. A determination of bacteriolysins showed that rats suffering from the lack of vitamin B develop bacteriolysins fully as well as animals receiving vitamin B and that rats lacking vitamin B do not suffer an increased susceptibility due to any inability to form bacteriolysins.

SUMMARY AND CONCLUSIONS

Assuming that there occurs in animals suffering from the lack of vitamins a break in the natural resistance offered to bacterial infection, wherein may the explanation of this cataphylaxis lie? It is quite generally accepted that metabolic processes taking place within an animal suffering from the lack of vitamins are greatly retarded. This being the case, it is but a short step to the assumption of a retarded antibody formation. To determine this, rats, rabbits and pigeons were fed suitable rations lacking vitamin A or B as decided on. After certain periods the animals showed striking symptoms of the dietary deficiency. They were then subjected to different treatments for the production of agglutinins, precipitins, hemolysins and bacteriolysins. Control animals received the same treatment.

Rats and rabbits lacking vitamin A or B showed no differences in their ability to produce agglutinins, precipitins, hemolysins or bacteriolysins. Similarly, the pigeon fed a diet of polished rice or a ration lacking vitamin B responded to the injection of antigen with the production of agglutinins.

It is therefore, reasonable to conclude that cataphylaxis in animals suffering from the lack of vitamins is not the result of the destruction or paralysis of the antibody forming mechanism that produces agglutinins, precipitins, hemolysins, or bacteriolysins.

IMMUNOLOGIC SIGNIFICANCE OF VITAMINS

II. INFLUENCE OF LACK OF VITAMINS ON RESISTANCE OF RAT, RABBIT AND PIGEON TO BACTERIAL INFECTION

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A break in the natural resistance of the animal body to bacterial infection has been suspected in animals suffering from the lack of vitamins. Renon¹ stated that a decrease in resistance to tuberculosis resulted on a vitamin poor diet. Muthu² believed in the significance of vitamins in tuberculosis. Frequently reports have been made of respiratory infections when the diet was poor in vitamins.³ Dutton⁴ believed the diet, especially the vitamins, played a part in leprosy.

Xerophthalmia or keratomalacia offers an interesting example of reduced resistance. Micro-organisms normally held at bay in animals on a complete diet, rapidly invade the eye tissues of an animal lacking vitamin A, destroy the cornea and cause loss of the eye. Xerophthalmia is ascribed to the lack of vitamin A and has been produced in the rat, mouse, rabbit⁵ guinea-pig⁶ and chicken.⁷ All rats lacking vitamin A do not develop this condition; a large majority do, however. Guerrini⁸ found that pigeons fed on a diet of polished rice became susceptible to anthrax.

The purpose of the work now reported was to determine the influence of vitamins A and B on the natural resistance of the rat, rabbit and pigeon to infection. The susceptibility of the rat and rabbit to vitamin A deficiency and of the pigeon to vitamin B deficiency led to their being chosen for the experiments. Inasmuch as both the rat and the pigeon are normally immune to anthrax, *Bacillus anthracis* was selected for inoculation. The rabbit also has considerable resistance to anthrax. A diplococcus, resembling the pneumococcus, isolated from the lungs of a moribund rat was employed also for inoculation.

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¹ Bull. gén. de therap., 1914, 168, p. 91.

² Brit. Med. Jour., 1920, 2, p. 160.

³ Fleming, Macaulay and Clark: Scurvy and Pneumonia in Southern Rhodesia, 1910; Brit. Med. Research Committee: Accessory Food Factors, Special Report Series No. 38, 1919.

⁴ Med. Press, 1920, 109, p. 313.

⁵ Nelson, Lamb and Heller: Jour. Phys., 1922, 59, p. 335.

⁶ Funk: The Vitamines, 1922, p. 382.

⁷ Guerrero and Conception, Phillip: Jour. Sc., 1920, 17, p. 99.

⁸ Ann. d'Igiene, 1921, 31, p. 596.

A ration of casein (18%), dextrin (74%), salt mixture (McCollum) (5%), and yeast (3%) was fed to rats to produce vitamin A deficiency. By substituting filtered butterfat for the yeast a ration was obtained lacking vitamin B, and this was fed to pigeons and rats. The ration suggested by Nelson⁵ is low in vitamin A and was fed to rabbits; it consists of white corn (65%), linseed oil meal (3%), ground oats (22%), casein (5%), tankage (3%), calcium carbonate (1%), and sodium chloride (1%). Control animals received the missing vitamin, in filtered butterfat in the case of vitamin A and in yeast in the case of vitamin B. Distilled water was supplied to all animals.

Leukocytes and erythrocytes were counted independently. Platelets were determined by the Buckman and Hallisley method. The coagulation time of the blood was observed by use of the Brodie-Russel-Boggs coagulometer. A standard 4 mm. platinum loopful of blood was removed from the femoral artery and placed on the glass cone of the coagulometer for observation. A lateral movement was chosen as the end point, and the result was always confirmed by quickly noting the presence of a clot. The time was taken by a stop-watch.

During the course of the experiments, respiratory infections were noted in rats lacking vitamin A. This apparent break in the resistance of the rat was more vividly brought to our attention when the tail injection of 0.2 c.c. of a living 18-hour broth culture of *Bacterium typhosum* caused the death of 3 of 4 rats lacking vitamin A, while none of the controls died. We have often isolated gram-negative coliform bacilli from the heart blood, liver and spleen of vitamin deficient animals and occasionally have obtained other bacteria, such as staphylococci. Our experience indicated a decrease in resistance of vitamin starved animals, although no sharp break was noted.

CATAPHYLAXIS IN THE RAT LACKING VITAMIN A

Inasmuch as the white rat is relatively immune to anthrax, it was interesting to determine the effect of the lack of vitamin A. If rats deficient in this vitamin showed a susceptibility, it would afford more definite conclusions than would be the case with normally susceptible animals. For this purpose 6 rats suffering from the lack of vitamin A and the same number of controls were selected, weighing approximately 100 gm. each. Inasmuch as vitamin starved animals lose weight rapidly, it was impossible to obtain test and control animals of the same weight and the same age. For this reason the ages of the animals

were largely ignored, and the weights chosen approximately equal. This did not in any way invalidate the results of the experiment; in fact, it afforded more definite conclusions for the reason that if the age of the animal is a factor involved in its resistance, it is undoubtedly in favor of the test animals. The younger controls would be more susceptible than the older test animals, disregarding, of course, their condition of vitamin deficiency. Each of the 12 rats was given 0.1 c.c. of a suspension of *Bacillus anthracis* intraperitoneally. This culture was known as No. 3 and 0.1 c.c. killed rabbits regularly in 48 hours.

TABLE 1

CATAPHYLAXIS IN THE RAT ON A DIET LACKING VITAMIN A AND INJECTED WITH
BACILLUS ANTHRACIS

Rat No.	Treat-ment	Weight in Gm.	Red Blood Cells in Millions	White Blood Cells	Platelets	Hemo-globin (Dare)	Coagu-lation Time	Rectal Temper-ature, F.	Results
1	Control	140	9.920	10,200	780,000	95	2' 45"	102.4	Lived
2	Control	90	9.200	8,400	860,000	88	3' 7"	102.6	Lived
3	Control	110	8.900	8,600	930,000	80	2' 55"	101.6	Lived
4	Control	80	9.436	9,100	840,000	93	2' 35"	101.6	Died in 10 hours; peritonitis; B. anthracis in spleen and liver
5	Control	95	8.950	10,200	1,000,000	89	3' 15"	102.0	Lived
6	Control	105	9.180	11,300	620,000	90	3' 40"	103.1	Lived
7	Test A	130	9.376	6,200	610,000	80	2' 45"	98.9	Died in 120 hours; anthrax
8	Test A	85	9.600	5,600	450,000	83	2' 55"	98.0	Died in 40 hours; anthrax
9	Test A	90	7.980	5,300	340,000	79	3' 10"	99.3	Died in 96 hours; anthrax
10	Test A	85	8.117	5,200	760,000	94	1' 45"	97.5	Died in 36 hours; anthrax
11	Test A	90	7.650	7,200	520,000	75	3' 5"	97.5	Died in 48 hours; anthrax
12	Test A	80	8.250	4,300	450,000	87	3' 35"	99.7	Died in 120 hours; anthrax

All of the 6 rats lacking vitamin A died within 120 hours, the first within 36 hours. The controls lived (table 1). Heavily encapsulated anthrax bacilli, were isolated from the livers and spleens of the test rats, and the lesions indicated that anthrax had caused death. The rectal temperature of the vitamin short animals was considerably below normal. The hemoglobin (Dare) was subnormal comparably to the drop in the number of erythrocytes. No difference was observed in the coagulation time of the blood of control and vitamin short rats, although a drop in the number of platelets was recorded. Three other series of 12 rats each were used, the results checking accurately with those given.

That increased susceptibility in the vitamin A deficient rats is not peculiar to anthrax was shown when a diplococcus isolated from the pleural fluid of a moribund rat was injected. The coccus was gram-positive, produced methemoglobin on blood-agar, but no definite zone of hemolysis, and was not agglutinated by pneumococcus serums, types

TABLE 2
CATAPHYLAXIS IN THE RAT ON A DIET LACKING VITAMIN A AND INJECTED WITH
0.1 C C PNEUMOCOCCUS

Rat No.	Treatment	Weight in Gm.	Red Blood Cells in Millions	White Blood Cells	Platelets	Hemoglobin (Dare)	Coagulation Time	Rectal Temperature, F.	Results
1	Test A	85	6.200	4,300	330,000	87	3' 45"	101.0	Died, 48 hours
2	Test A	70	6.760	11,200	670,000	91	2' 50"	97.5	Died, 48 hours
3	Test A	65	11.450	9,200	780,000	109	2' 38"	99.3	Died, 72 hours
4	Test A	90	12.340	8,400	470,000	120+	3' 54"	99.1	Died
5	Test A	85	7.440	5,100	540,000	83	2' 37"	98.6	Lived; recovered on complete ration
6	Test A	90	8.760	7,300	820,000	92	3' 17"	100.7	Gradually recovered after two weeks
7	Control	95	8.900	10,200	780,000	91	2' 17"	102.5	Lived
8	Control	110	9.180	8,400	630,000	96	3' 52"	100.3	Lived
9	Control	85	8.990	13,300	920,000	94	1' 50"	102.9	Lived
10	Control	110	7.670	10,400	845,000	98	3' 45"	102.5	Died; mixed peritonitis
11	Control	120	9.350	8,900	880,000	96	3' 57"	101.9	Lived
12	Control	100	9.160	7,800	650,000	101	2' 12"	102.1	Lived

TABLE 3
CATAPHYLAXIS IN THE RABBIT ON A DIET DEFICIENT IN VITAMIN A AND INJECTED WITH
BACILLUS ANTHRACIS

Rabbit No.	Treatment	Rectal Temperature, F.	Weight in Gm.	Bacterium anthracis, C c.	Results
1	Test A	99.8	2,135	0.07	Died, 72 hours; anthrax
2	Test A	100.6	1,985	0.07	Died, 120 hours; anthrax
3	Test A	101.3	2,260	0.07	Lived
4	Test A	98.9	2,350	0.08	Died, 48 hours; anthrax
5	Test A	98.4	2,155	0.07	Died, 24 hours; anthrax
6	Test A	101.8	2,475	0.09	Lived
7	Control	105.2	2,375	0.08	Lived
8	Control	103.1	2,220	0.07	Lived
9	Control	104.3	2,460	0.09	Died, 130 hours; anthrax
10	Control	104.1	2,570	0.10	Lived

1, 2, or 3. It was highly fatal to white mice, and virulence was maintained by preserving the livers of mice which had died from the infection.

Six rats suffering from the lack of vitamin A and 6 controls of approximately the same weight were injected intraperitoneally each with

0.1 c c. of a supernatant suspension from macerating an infected mouse liver in salt solution. Table 2 shows that 4 of the 6 test animals died within 72 hours, 2 finally recovered on a complete diet, whereas of the control rats 5 remained alive and 1 died from peritonitis. These results consistently obtained show the increase in susceptibility resulting in vitamin starved animals. This increase in susceptibility seems comparable to a starvation cataphylaxis. The test rats gave lower rectal temperature and reduced cell counts. Two other similar series gave similar results.

The varied effect of vitamin A deficiency on the rat and rabbit must be borne in mind. The rectal temperature and cell counts of vitamin A starved rats may remain normal for surprisingly long periods. Temporary polycythemia is by no means rare and may occur as a relative condition in rats early in the feeding period. A decrease in erythrocytes usually appears, however, after prolonged feeding, although rats often die without oligocythemia. The leukocytes and the platelets after prolonged feeding quite regularly show a decrease. The hemoglobin follows quite closely the red blood cell count. The coagulation time of the blood of the test rats did not differ from that of the controls. The hematology of avitaminosis will be discussed more completely in a later paper.

CATAPHYLAXIS IN RABBITS ON A DIET DEFICIENT IN VITAMIN A

The resistance of the rabbit to bacterial infection is likewise lowered when vitamin A is withheld. The rabbits in table 3 suffered from a severe avitaminosis and had received the vitamin A low diet for 14 weeks. Each rabbit received intraperitoneally 0.07 to 0.10 c c. of a thin suspension of a culture of *Bacillus anthracis* having a virulence sufficient to kill guinea-pigs in 0.1 c c. injections, but not regularly fatal to rabbits. Four of the 6 test animals died from the inoculation, the last in 120 hours. Two remained alive. One control died with anthrax in 130 hours, the other 3 controls lived. It is to be noted that the rectal temperature of the test animals was considerably below normal.

CATAPHYLAXIS IN THE PIGEON ON A DIET LACKING VITAMIN B

The culture of *Bacillus anthracis* in this experiment killed mice and guinea-pigs but not rabbits regularly, when injected intraperitoneally in 0.1 c c. amounts. The break in the resistance of rice fed

polyneuritic pigeons is quite definite (table 4). The results agree essentially with those of Guerrini.⁸ The extent to which the nervous system may control immunity is not clear. As paralysis is quite general in vitamin B deficiency, the nervous system may be of real significance in immunologic relationships. Lower rectal temperatures were recorded in the test pigeons, likewise leukopenia. Both were probably related to the break in resistance.

TABLE 4
CATAPHYLAXIS IN THE PIGEON ON A DIET OF POLISHED RICE AND INJECTED WITH 0.1 C.C. OF *B. ANTHRACIS*

Pigeon No.	Treatment	Weight in Gm.	Rectal Temperature, F.	Results
1	Rice diet	150	99.8	Died, 48 hours; anthrax
2	Rice diet	135	97.5	Died, 24 hours; anthrax
3	Rice diet	140	100.4	Died, 120 hours; anthrax
4	Rice diet	155	103.1	Died, 120 hours; anthrax
5	Rice diet	160	101.1	Died, 72 hours; anthrax
6	Control	170	106.9	Lived
7	Control	165	109.6	Lived
8	Control	160	107.8	Lived
9	Control	145	108.7	Lived
10	Control	150	110.0	Lived

TABLE 5
CATAPHYLAXIS IN THE PIGEON ON A DIET LACKING VITAMIN B. AND INJECTED WITH 0.1 C.C. *BACILLUS ANTHRACIS*

Pigeon No.	Treatment	Weight in Gm.	Rectal Temperature, F.	Results
1	Test B	145	103.8	Died, 36 hours; anthrax
2	Test B	150	101.6	Died, 48 hours; anthrax
3	Test B	165	100.2	Died, 24 hours; anthrax
4	Test B	145	98.7	Died, 30 hours; anthrax
5	Test B	155	99.4	Died, 72 hours; anthrax
6	Control	175	106.7	Lived
7	Control	165	107.8	Lived
8	Control	180	109.6	Lived
9	Control	170	109.6	Lived

Similar results were obtained in pigeons fed on a diet lacking only vitamin B. The test pigeons failed to survive an injection of 0.1 c.c. of a suspension of *Bacillus anthracis* similar to that used in the preceding experiment. The controls inoculated with 0.1 c.c. remained alive (table 5).

Table 6 shows essentially the same break in the resistance of the pigeon to pneumococci. The lower rectal temperature of the test pigeons is noteworthy.

Just what the effect of a reduced body temperature has on the immunity mechanism is not clear. It apparently plays little part in the production of antibodies, as no differences were observed in the production of agglutinins, hemolysins or bacteriolysins in vitamin starved animals.⁹ In animals dying with anthrax or pneumococcus

TABLE 6

CATAPHYLAXIS IN THE PIGEON ON A DIET LACKING VITAMIN B AND INJECTED WITH 0.12 C.C. PNEUMOCOCCUS

Pigeon No.	Treatment	Weight in Gm.	Rectal Temperature, F.	Results
1	Test B	145	97.8	Died, 24 hours
2	Test B	150	101.6	Lived
3	Test B	130	99.4	Died, 48 hours
4	Test B	165	102.6	Lived
5	Test B	140	101.5	Died, 48 hours
6	Control	165	107.5	Lived
7	Control	160	108.4	Lived
8	Control	180	108.9	Lived
9	Control	150	107.9	Lived
10	Control	145	109.3	Lived

TABLE 7

CATAPHYLAXIS IN THE RAT LACKING VITAMIN B AND INJECTED WITH BACILLUS ANTHRACIS

Rat No.	Treatment	Weight in Gm.	Rectal Temperature, F.	White Blood Cells	Red Blood Cells in Millions	Platelets	Coagulation Time	Results
1	Test B	85	97.5	4,200	11.20	840,000	2' 37"	Lived
2	Test B	65	99.6	6,500	7.450	640,000	2' 15"	Died, 48 hours
3	Test B	60	98.7	7,300	6.700	430,000	3' 44"	Died, 24 hours
4	Test B	55	100.3	8,600	8.760	870,000	2' 30"	Died, 90 hours
5	Test B	40	98.6	4,600	7.230	360,000	3' 30"	Died, 36 hours
6	Test B	65	99.4	7,800	5.970	580,000	2' 37"	Died, 48 hours
7	Test B	70	99.3	7,400	9.100	940,000	2' 5"	Lived
8	Test B	55	96.6	2,400	2.300	290,000	2' 47"	Died, 24 hours
9	Test B	50	99.7	10,200	8.600	860,000	3' 40"	Died, 72 hours
10	Test B	60	99.0	6,400	9.900	830,000	3' 15"	Lived
11	Test B	65	100.7	4,900	10.350	910,000	1' 40"	Lived
12	Control	70	102.7	9,200	8.240	1,100,000	2' 10"	Lived
13	Control	65	103.3	9,500	7.890	960,000	1' 55"	Lived
14	Control	55	103.1	15,900	8.240	1,300,000	2' 55"	Lived
15	Control	75	103.1	9,100	9.440	780,000	2' 35"	Lived
16	Control	80	102.9	7,800	9.960	860,000	3' 15"	Died, 72 hours

infection, large capsules of the invading organism were noted, and it is evident that phagocytic ingestion does not keep pace with the growth and reproduction of the bacteria. After a short time the phagocytes migrate from the peritoneal cavity, the heavily encapsulated organisms flourish, and the death of the animal ensues. In the control animals

⁹ Werkman, C. H.: Jour. Infect. Dis., 1923, 32, p. 253.

a reduction in numbers of bacteria occurs promptly, and the peritoneal fluid reveals few, if any, organisms.

CATAPHYLAXIS IN THE RAT LACKING VITAMIN B

It is not to be inferred that a break in resistance is a specific result of vitamin A deficiency. Table 7 refers to a series of 11 rats lacking vitamin B and 5 controls. Each of the 16 rats received 1.25 c.c. of a suspension of *Bacillus anthracis* which killed guinea-pigs (0.1 c.c. intraperitoneally) but rarely rabbits. Seven of the 11 test B animals died, the last after 90 hours. One of the 5 controls succumbed after 72 hours. Red blood cells, leukocytes and platelets were reduced in number. There were no changes in the time of coagulation. A lower rectal temperature of the test B rats is noted.

SUMMARY

Rats, rabbits and pigeons suffering from pronounced vitamin deficiencies suffered a marked break in their resistance to infection. Rats and rabbits lacking vitamin A became less resistant to infection with the anthrax bacillus and the pneumococcus. As rats suffering from the lack of vitamin B likewise developed an increase in susceptibility to the anthrax bacillus and pneumococcus, the cataphylaxis is not peculiar to vitamin A deficiency. The results are similar to starvation susceptibility.

Pigeons fed on a diet lacking vitamin B only, and pigeons fed on a diet of polished rice, readily succumbed to infection with the anthrax bacillus and the pneumococcus, while the control pigeons survived.

IMMUNOLOGIC SIGNIFICANCE OF VITAMINS

III. INFLUENCE OF THE LACK OF VITAMINS ON THE LEUKOCYTES AND ON PHAGOCYTOSIS

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Previous investigations have shown that rats, rabbits and pigeons suffering from the lack of certain vitamins experience a marked break in their resistance to infection,¹ but that this cataphylaxis is not the result of any inhibition in the formation of agglutinin, precipitin, hemolysin or bacteriolysin.² As the prevalence in vitamin-starved animals of a type of infection normally suppressed by phagocytic activity has been previously pointed out, a study of the behavior of the phagocytes assumes particular importance.

Findlay and MacKenzie³ recently concluded that the normal phagocytic activity in vitamin-starved rats is not depressed. Their tests were made in vitro, and they did not determine the phagocytic index in immune animals. Other investigators have shown that the production of opsonins is a relatively stable process. Hektoen and Curtis⁴ and Hektoen⁵ have shown that the removal of the spleen, thyroid, small intestine or stomach of the dog does not interfere with antibody formation (opsonins for rat corpuscles). Bordet⁶ and Cross⁷ also have shown the stability of the capacity of the body to produce opsonins.

On the other hand, Sanarelli⁸ found that the phagocytes from chilled guinea-pigs were less active for *Sp. metchnikovii* than those of controls. Dean⁹ found that the opsonic activity was nearly 10 times greater at 37 C. than at 6 C. Rolly and Meltzer¹⁰ showed that the activity of the phagocytes increased gradually from 6 C. to 37.5 C. and

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¹ Werkman, C. H.: *Jour. Infect. Dis.*, 1923, 32, p. 260.

² *Ibid.*, p. 247.

³ *Biochem. Jour.*, 1922, 16, p. 574.

⁴ *Jour. Infect Dis.*, 1915, 17, p. 409.

⁵ *Ibid.*, 1920, 27, p. 23.

⁶ *Studies in Immunity*, 1921.

⁷ *Bull. Johns Hopkins Hosp.*, 1921, 32, p. 350.

⁸ *Ann. de l'Inst. Pasteur*, 1893, 7, p. 225.

⁹ *Proc. Roy. Soc.*, 1905, 76, p. 706.

¹⁰ *Deutsch. Arch. f. klin. Med.*, 1908, 94, p. 335.

markedly from 37.5 C. to 40 C. Trommsdorff¹¹ and Ledingham¹² also have shown the influence of temperature on phagocytic activity.

The present investigation deals with the phagocytic activity in normal and immune rats and rabbits lacking vitamin A or B. Experiments were carried out in vitro and in vivo using the same strain of *Bacterium typhosum* (typhoid bacillus). *Staph. albus* was employed in one experiment. Leukocytes for the tests in vitro were obtained from two or more stock animals by peptone injections and pooled.

Rats lacking vitamin A were fed on a diet of dextrin (74%), casein (18%), yeast (3%), and salt mixture of McCollum (5%). The ration lacking vitamin B was similar to the above ration with butterfat in place of yeast. Rabbits lacking vitamin A were fed on the ration suggested by Nelson.¹³ Controls received butterfat or yeast to supply the missing vitamin. All animals received distilled water.

The method of Wright¹⁴ was used in the opsonic determination in vitro. The determinations in vivo were carried out in the following manner:

Twelve hours previous to the determinations, each rat received intraperitoneally 1.0 c.c. sterile 20% peptone solution. At the time of test each animal was injected intraperitoneally with approximately 1.0 c.c. of an 18-hour broth culture of *Bacterium typhosum*. The amount was varied to some extent depending on the size of the animal. After an interval of 15 minutes smears were made from the material obtained in a Wright pipet by peritoneal puncture. The ratio of the leukocytes to bacteria was always determined by examining a stained smear and counting both bacteria and leukocytes in a given area, care always being taken that at least 100 leukocytes were counted. The animal was discarded if the ratio of leukocytes to bacteria was not within the limits of 1/4 to 1/9 for *Bacterium typhosum*. The average number of bacteria ingested per leukocyte was determined for 100 leukocytes.

PHAGOCYTIC ACTIVITY IN THE RAT AND RABBIT LACKING VITAMIN A

The phagocytic index of the pooled serums of 3 nonimmunized rats lacking vitamin A and 3 control rats that had received the vitamin were determined in vitro according to the technic of Wright. The 3 animals lacking vitamin A showed xerophthalmia and were in poor condition at the time of bleeding. They had been on the vitamin deficient ration for 6 weeks and would probably have died within the following week. Leukocytes for the determinations were obtained from ordinary stock rats and the serums to be tested was obtained by cardiac puncture.

¹¹ Arch. f. Hyg., 1906, 59, p. 1.

¹² Proc. Roy. Soc., 1908, 80, p. 188.

¹³ Jour. Physiol., 1922, 59, p. 335.

¹⁴ Kolmer: Infection, Immunity and Specific Therapy, 1917.

Two organisms were employed: *Bacterium typhosum* and *Staph. albus*. The phagocytic index of the test serum was for *Bact. typhosum*, 0.97; for *Staph. albus*, 1.43; for the control serum for *Bact. typhosum*, 1.10; for *Staph. albus*, 1.61. The ratio of leukocytes to bacteria (*Bact. typhosum*) in the case of the control serum was slightly greater ($\frac{1}{9}$) than in the test serum ($\frac{1}{7}$); the same held true for *Staph. albus* ($\frac{1}{14}$ and $\frac{1}{11}$). The results show no significant differences between the opsonic activity of test and control serums.

Opsonic indexes were determined for 2 groups of rabbits at weekly intervals for 4 consecutive weeks. At the time of the first determination one group of 4 rabbits had received a ration low in vitamin A for 12 weeks and 2 animals in this group subsequently developed

TABLE 1

PHAGOCYTIC ACTIVITY IN THE NONIMMUNIZED RAT LACKING VITAMIN A IN VIVO

Rats	Treatment	Ratio of Leukocytes to Bacteria	Percentage of Leukocytes Ingesting Various Numbers of Bacteria				Phagocytic Index
			0	1	2	3 or More	
1	Test A	1:7	58	17	15	10	0.78
2	Test A	1:5	49	23	21	7	0.87
3	Test A	1:5	44	21	28	7	1.07
4	Test A	1:6	54	23	19	4	0.79
Average.....			51.2	21.0	20.8	7.0	0.88
5	Control	1:8	40	19	31	10	1.03
6	Control	1:5	51	20	22	7	0.87
7	Control	1:7	47	21	26	6	0.98
8	Control	1:8	42	18	23	17	1.22
Average.....			45	19.5	25.5	10.0	1.03

xerophthalmia; the other group of 4 rabbits received a complete ration and were in good condition. Serum was obtained from the marginal vein of the ear, and leukocytes were obtained from stock rabbits. The phagocytic indexes of the pooled serum for the test group were during consecutive weeks: 0.86, 1.23, 0.91 and 0.99; for the control group: 0.98, 0.87, 1.31, and 1.10.

A determination of the opsonic indexes of the serum from non-immunized animals in vitro offers therefore no explanation for the marked cataphylaxis experienced by animals suffering from the lack of vitamin A.

In table 1 are given the results of an experiment in vivo on the phagocytic activity of rat leukocytes from animals lacking vitamin A. In this as in subsequent experiments there was a somewhat well defined tendency for the control serums to show a greater average ingestion.

Especially the number of leukocytes failing to ingest any bacteria was reduced in the control animals. The 4 test rats in table 1 were suffering from advanced avitaminosis and would undoubtedly have died within a few days. The significance of these results in their relation to vitamin cataphylaxis is not marked although, in a measure, reduced phagocytic activity may contribute to the break in the resistance of avitaminic animals to infection. The leukocytes of vitamin-starved animals may be subject to fundamental alterations. Leukopenia is a common result of vitamin deficiency and as the body temperatures are considerably lowered, the effect on phagocytic activity may be of considerable significance. The reduced metabolism of vitamin deficient animals may extend to the phagocytic functioning of their leukocytes. Hess¹⁵ and de Wyss,¹⁶ among others, have shown that the enzymes of tissue respiration are reduced and oxidative processes lessened in avitaminic animals, whereas, Arkin¹⁷ found that phagocytic activity was stimulated by increased oxidative changes.

An experiment was made *in vitro* to determine the phagocytic indexes of the serum of immunized rats lacking vitamin A and immunized controls. The animals received intraperitoneal injections of 0.2, 0.3, 0.4, 0.6 and 1.0 c.c. severally at weekly intervals of an 18-hour broth culture of *Bacterium typhosum*. The rats had been on diet 4 weeks when they received their first injection. The phagocytic index was then determined one week after the last injection. Quite a few of the test rats died during the experiment and results were obtained only for 3 survivors. The ratio of the number of leukocytes to bacteria was kept within the limits of 1/4 to 1/9.

The phagocytic indexes for the test animals were 4.65, 5.09, 4.36; and for the controls, 4.27, 4.60 and 5.08. Normal rat serum serving as a check was 0.87. From these results there is no evidence of a decrease in the ability of vitamin deficient animals to produce immune opsonins.

An application of the opsonic index assumes necessarily that the conditions of the test are representative of conditions *in vivo*. This is known not to be true, as the test measures the activity of opsonin, whereas *in vivo* there exist innumerable factors—known and unknown—which may fundamentally influence the result of phagocytic activity. Temperature has already been shown to exert considerable influence; and the part played by the leukocytes themselves in diseased and in healthy conditions has received little study.

¹⁵ Ztschr. f. physiol. Chem., 1921, 117, p. 284

¹⁶ Lancet, 1922, 1, p. 100.

¹⁷ Jour. Infect. Dis., 1913, 13, p. 418.

It is desirable to point out here certain temperature relationships. It is known that the optimum temperature for the development of virulence by many of the pathogens is 37 C. (98.6 F.), and at temperatures slightly above this the growth and virulence of the organism may suffer considerably. As the normal rectal temperatures of the laboratory animals employed average somewhat higher than the optimum for exaltation of the pathogens, the lowered temperatures occurring in avitaminic animals actually favor the growth and development of virulence in the organism.

Table 2 summarizes an experiment *in vivo* to determine the phagocytic activity in immunized rats deficient in vitamin A and immunized controls. The animals received the same treatment as those in the fore-

TABLE 2
PHAGOCYTIC ACTIVITY IN THE IMMUNIZED RAT LACKING VITAMIN A *IN VIVO*

Rats	Treatment	Ratio of Leuko- cytes to Bacteria	Percentage of Leukocytes Ingesting Various Numbers of Bacteria									Phago- cytic Index
			0	1	2	3	4	5	6	7	8 or more	
1	Test A	1:7	11	6	13	19	14	12	13	8	4	3.75
2	Test A	1:5	21	16	16	14	7	9	4	6	7	2.97
3	Test A	1:6	17	12	19	16	13	11	7	2	3	2.88
	Average..	16.3	11.3	16.0	16.3	11.3	10.6	8.0	5.3	4.6	3.20
4	Control	1:7	7	4	13	11	8	21	14	11	11	4.81
5	Control	1:8	6	7	14	21	17	8	11	3	13	4.10
6	Control	1:4	11	5	11	14	17	14	9	10	9	4.12
	Average..	8.0	5.3	12.6	15.3	14.0	14.3	11.3	8.0	11.0	4.34
7	Normal stock serum.....											0.64

going experiment but were bled on the ninth day after the last injection. The phagocytic indexes of the test rats average considerably below those of the controls. The percentages of inactive leukocytes average less in the control rats than in the test animals. It would appear that an inhibiting influence is active in depressing phagocytic activity in vitamin-starved animals.

PHAGOCYTIC ACTIVITY IN THE RAT LACKING VITAMIN B

In view of the phagocytic behavior of animals lacking vitamin A, similar experiments were carried out using rats lacking vitamin B. It was not thought that depressed phagocytic activity specifically resulted when vitamin A was withheld from the diet. The general depression of resistance of vitamin starved animals may be a part of the general metabolic depression which results during starvation. Vitamin cata-

phylaxis may be placed in the same category with starvation cataphylaxis. The conditions are, however, much more severe in experimental animals lacking vitamins than would probably occur in human pathology.

The determination of the phagocytic indexes of nonimmune rat serums deficient in vitamin B was carried out with 4 animals which had received the vitamin B-free diet for 11 weeks. Three controls

TABLE 3
PHAGOCYTIC ACTIVITY IN THE IMMUNIZED RAT LACKING VITAMIN B IN VITRO

Rats	Treatment	Ratio of Leuko- cytes to Bacteria	Percentage of Leukocytes Ingesting Various Numbers of Bacteria									Phago- cytic Index
			0	1	2	3	4	5	6	7	8 or more	
1	Test B	1:7	7	6	14	22	21	5	9	5	11	4.13
2	Test B	1:5	11	6	12	19	23	11	8	3	7	3.93
3	Test B	1:6	3	4	9	9	18	21	10	13	13	4.96
	Average..	7.0	5.3	11.6	16.6	20.6	12.3	9.0	7.0	10.3	4.34
4	Control	1:6	6	3	11	14	9	19	17	8	13	4.84
5	Control	1:8	7	6	7	9	11	14	24	10	12	4.97
6	Control	1:7	4	4	9	11	14	27	13	8	11	4.78
	Average..	5.6	4.3	9.0	11.3	11.3	20.0	18.0	8.6	12.0	4.86
7	Normal stock serum.....											0.73

TABLE 4
PHAGOCYTIC ACTIVITY IN THE IMMUNIZED RAT LACKING VITAMIN B IN VIVO

Rats	Treatment	Ratio of Leuko- cytes to Bacteria	Percentage of Leukocytes Ingesting Various Numbers of Bacteria									Phago- cytic Index
			0	1	2	3	4	5	6	7	8 or more	
1	Test B	1:6	13	8	14	17	11	11	9	9	8	3.75
2	Test B	1:5	17	14	15	16	15	9	3	6	4	2.93
3	Test B	1:5	19	11	17	19	13	8	8	4	1	2.79
	Average..	16.3	11.0	15.3	17.3	13.0	9.3	6.6	6.3	4.3	3.16
4	Control	1:5	6	7	8	11	14	17	14	11	12	4.78
5	Control	1:6	5	8	15	19	18	10	11	8	6	4.15
6	Control	1:7	11	4	12	16	11	13	8	10	12	4.43
	Average..	7.3	6.3	11.6	15.3	15.3	13.3	11.0	9.6	10.0	4.45
7	Normal stock serum (ratio of leukocytes to bacteria, 1:6).....											0.73

were employed. The phagocytic indexes in vitro for the test rats were: 1.23, 1.32, 0.89 and 1.00; for the controls, 1.31, 0.88 and 0.98. No depression in the test animals occurred. Determined in vivo, the phagocytic activities were for the same animals within 48 hours later: 0.69, 0.87, 1.07 and 0.79; for the controls: 1.06, 0.98 and 1.22. The significance of the differences is problematical.

Table 3 has reference to the immune opsonins determined in vitro. Three rats that had received no vitamin B for 12 weeks were given intraperitoneal injections of 0.2, 0.3, 0.5, 1.0 and 1.2 c.c. of a killed 18-hour broth culture of *Bacterium typhosum* at weekly intervals. Three controls received the same injections. Opsonic indexes were determined 10 days after the last injection. There is no evidence of subnormal values for the test animals.

Determined in vivo the results in vitamin B deficiency again show a lower phagocytic activity than was found in the controls. The three test animals were from the same group as those in table 3 and had received the same treatment and same injections but were tested for phagocytic activity one week after the last injection. The results in table 4 show a depressed activity of the phagocytes in vitamin B deficient animals. The differences are not great.

SUMMARY AND CONCLUSIONS

The presence in animals lacking vitamins of a type of infection normally suppressed by phagocytic activity led to the present investigation of phagocytic activities in rats and rabbits lacking vitamin A or vitamin B. The normal opsonic indexes determined in vitro for *Bact. typhosum* and *Staph. albus* in rats and rabbits lacking vitamin A were not appreciably depressed. Similarly, the phagocytic indexes of rats lacking vitamin B for *Bact. typhosum* did not exhibit marked variations from those in the control animals. A determination of the phagocytic indexes in immune rats in vitro likewise revealed no differences to account for vitamin cataphylaxis.

When determined in vivo with *Bacterium typhosum*, the results were somewhat more striking. The indexes were slightly greater in the rat in nonimmunized controls than in nonimmunized rats suffering from the lack of vitamin A or vitamin B. As the differences were small, their significance from the standpoint of disease resistance is not to be overemphasized. Similarly, in the immunized animals there was a consistent difference in favor of the control animals. These results appear sufficient to be significant in vitamin cataphylaxis. Considered in the light of results obtained in vitro, they suggest that the depression of the phagocytic activity does not result through a failure of the animal to elaborate opsonins, but as the result of a depressive agent acting on the phagocytic mechanism. Temperature may be of significance in this respect, as the body temperature is greatly lowered during vitamin starvation.

GAS PRODUCTION BY BACTERIA IN SYMBIOSIS

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There seem to have been few instances observed of the production by bacteria in association of substances which none of the associated organisms alone can produce from the same medium. Nencki¹ mentions the formation from glucose of normal butyl alcohol by mixtures of *B. paralactici* and *B. chauvœi*, neither of which in pure culture produces this substance. Burri and Stutzer² observed the production of free nitrogen from sodium nitrate by mixtures of *B. coli* and *B. denitrificans*. In pure culture neither of these organisms can reduce nitrates to free nitrogen. Recently Knorr³ reported that certain foul odors are produced in liver broth by the symbiotic action of *Fusobacterium* and a streptococcus, while pure cultures of each organism on the same medium were entirely odorless. This author also observed the formation of gas in the liver medium by the combined action of *Fusobacterium*, *Sp. sputigenum* and streptococci. When streptococci were absent, no gas was formed.

Through some observations made in connection with another research, we had been led to believe that the production of gas in carbohydrate mediums might be brought about by the joint action of two or more organisms not by themselves capable of producing gas on the sugar used. Accordingly, a number of tests were made with mixtures of the ordinary laboratory strains, using lactose, saccharose and mannite as the fermentable substances. The result was that a considerable number of gas-forming pairs were discovered. Table 1 gives the positive results of these tests. On account of a generalization which we were able to formulate from our experiments, it is not considered necessary to record the negative findings.

The medium used had a meat extract peptone base and contained 1% of the fermentable substance. The reaction was adjusted to P_H 7. Pfannstiehl sugars were employed in all tests, and proper controls were made to rule out possible impurities or partial decomposition of

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¹ Centralbl. f. Bakteriöl., 1892, 11, pp. 225.

² Ibid., 1894, 16, p. 815.

³ Ibid., 1922, I., O., 87, p. 536.

the sugars in the process of sterilization. Five inch Smith fermentation tubes were used, and inoculation was made by transferring one loopful of each species from a 24-hour pure culture in the same medium.

Numerous repetitions of the tests of table 1 were made at different times and with different batches of medium, and it was found that the gas percentages obtained varied within wide limits. The figures given are from one test only. Though in some cases the amount of gas produced by a given pair of organisms ran very low, at no time did

TABLE 1

GAS PRODUCTION BY PAIRS OF ORGANISMS INCUBATED TOGETHER IN SUGAR PEPTONE BROTH

Organisms Inoculated		Sugar	% Gas in 5 Days at 37 C.
<i>B. prodigiosus</i>	+ <i>B. cholerae suis</i>	Lactose	12
<i>Staphylococcus aureus</i> +	<i>B. paratyph. B.</i>	Lactose	5
<i>Streptococcus fecalis</i> ..	+ <i>B. cholerae suis</i>	Lactose	5
<i>Strep. fecalis</i>	+ <i>B. paratyph. B.</i>	Lactose	30
<i>Strep. fecalis</i>	+ <i>B. pneumoniae</i>	Lactose	24
<i>Staph. aureus</i>	+ <i>B. proteus vulg.</i>	Lactose	5
<i>Strep. fecalis</i>	+ <i>B. morgani</i>	Lactose	5
<i>B. butyricus</i>	+ <i>B. proteus vulg.</i>	Lactose	10
<i>Vibrio proteus</i>	+ <i>B. proteus vulg.</i>	Lactose	5
<i>B. lautus</i>	+ <i>B. proteus vulg.</i>	Lactose	10
<i>B. prausnitzii</i>	+ <i>B. proteus vulg.</i>	Lactose	10
<i>Staph. aureus</i>	+ <i>B. coli communis</i>	Saccharose	10
<i>Strep. fecalis</i>	+ <i>B. coli communis</i>	Saccharose	12
<i>B. butyricus</i>	+ <i>B. coli communis</i>	Saccharose	22
<i>Sp. metchnikovi</i>	+ <i>B. paratyph. B.</i>	Saccharose	10
<i>Sp. cholerae</i>	+ <i>B. paratyph. B.</i>	Saccharose	9
<i>Staph. aureus</i>	+ <i>B. ieteroides</i>	Saccharose	25
<i>B. viscosum</i>	+ <i>B. cholerae suis</i>	Saccharose	10
<i>B. prodigiosus</i>	+ <i>B. cholerae suis</i>	Saccharose	10
<i>Strep. fecalis</i>	+ <i>B. acidi lactici</i>	Saccharose	15
<i>Staph. aureus</i>	+ <i>B. proteus vulg.</i>	Mannite	12
<i>Strep. fecalis</i>	+ <i>B. proteus vulg.</i>	Mannite	16
<i>Strep. hemolyticus</i>	+ <i>B. proteus vulg.</i>	Mannite	26
<i>Strep. viridans</i>	+ <i>B. proteus vulg.</i>	Mannite	50
<i>B. dysenteriae, Flexner</i>	+ <i>B. proteus vulg.</i>	Mannite	20
<i>B. dysenteriae Y.</i>	+ <i>B. proteus vulg.</i>	Mannite	12
<i>B. typhosus</i>	+ <i>B. proteus vulg.</i>	Mannite	24
<i>B. centrosporus</i>	+ <i>B. proteus vulg.</i>	Mannite	9

we find that a pair giving gas on one occasion would completely fail to produce gas on other occasions, provided the conditions were kept as nearly the same as possible. The ability to produce gas from a given sugar seemed to be a constant quality of the combination.

The experiments described were carried out using a single strain of each species. The question then naturally arose whether the ability to form a gas-producing complex with any given organism is a characteristic of a species or whether there are individual variations in this quality. The data in table 2 answer this question in part. It will be observed that all strains reacted regularly, except *B. paratyphosus* A 152. In the case of this organism in combination with *streptococcus*

G3, repeated trials failed to elicit any trace of gas formation. So far as our experiments go, therefore, we can say only that an occasional strain is encountered which fails to react in the same way as the majority.

All the streptococcus strains of table 2 were *Streptococcus fecalis* according to Holman's classification. The paratyphoid strains and proteus strains 6 and 213 were collection strains of unknown origin. All other proteus strains were recently isolated from infant stools. All cultures were proved to be morphologically and culturally typical, and the paratyphoid strains were known to be serologically true to type.

TABLE 2
GAS PRODUCTION BY PAIRS OF DIFFERENT BACTERIAL STRAINS

Organisms Inoculated	Sugar	% Gas in 5 Days
Strep. G ₃ + B. paratyph. B 162.....	Lactose	5
Strep. G ₃ + B. paratyph. B 163.....	Lactose	5
Strep. G ₃ + B. paratyph. B 164.....	Lactose	35
Strep. G ₃ + B. paratyph. B 165.....	Lactose	40
Strep. G ₃ + B. paratyph. A 152.....	Lactose	None
Strep. G ₃ + B. paratyph. A 153.....	Lactose	30
Strep. G ₃ + B. paratyph. A 154.....	Lactose	25
Strep. N ₁ + B. proteus A ₃	Mannite	20
Strep. N ₁ + B. proteus I ₃	Mannite	25
Strep. N ₁ + B. proteus 213.....	Mannite	25
Strep. N ₁ + B. proteus L ₂	Mannite	20
Strep. N ₁ + B. proteus 6.....	Mannite	15
Strep. N ₁ + B. proteus I ₂	Fructose	10
Strep. G ₃ + B. proteus L ₂	Fructose	9
Strep. V ₁ + B. proteus L ₂	Fructose	12
Strep. O ₁ + B. proteus L ₂	Fructose	4

CONDITIONS FAVORING GAS FORMATION BY A SYMBIOTIC
PAIR OF ORGANISMS

For the study of the effect of the conditions of inoculation and composition of the medium on the rapidity and amount of gas formation, a single symbiotic pair was chosen, *Streptococcus fecalis* N₁ and *B. proteus-vulgaris* L₂. Both of these strains had been isolated recently from infant stools. Mannite was used as the fermentable substance. The proteus strain was incapable of attacking this substance alone. The streptococcus fermented it readily with acid formation. The progressive change in the reaction of the medium due to the growth of each organism separately and to that of the two together is shown in table 3. The method used here was to inoculate about 50 c.c. of the medium in small flasks with the specified organisms and then withdraw at intervals a small quantity, 5 c.c., and determine the hydrogen-ion

concentration by the method of Medalia.⁴ It is seen that the streptococcus rapidly produces a high acidity and that the proteus strain produces an initial increase of the hydrogen-ion concentration followed by a gradual lowering until a decidedly alkaline reaction is reached. This P_H curve is characteristic of *B. proteus* in a medium in which no fermentable substance is present. The change in reaction in the medium

TABLE 3
CHANGE IN HYDROGEN-ION CONCENTRATION IN 1% MANNITE BROTH

Culture Inoculated	P _H after Incubation for							% Gas after 1 Week
	4 Hrs.	6 Hrs.	8 Hrs.	10 Hrs.	27 Hrs.	33 hrs.	1 Week	
Strep. N ₁	6.9	6.8	6.0	5.4	4.7	4.6	4.4	None
Proteus L ₂	6.9	6.6	6.4	6.6	7.3	7.4	8.1	None
Strep. N ₁ + Proteus L ₂	6.9	6.5	6.2	5.7	6.0	6.0	5.1	20%

TABLE 4
INFLUENCE OF THE RELATIVE NUMBERS INOCULATED ON THE AMOUNT OF GAS PRODUCED BY TWO ORGANISMS IN SYMBIOSIS

Tube No.	Estimated Number of Organisms Inoculated		Ratio	% Gas in 10 Days
	Streptococcus N ₁	B. proteus L ₂		
1.....	100	300,000,000	1 to 3,000,000	57
2.....	1,000	300,000,000	1 to 300,000	45
3.....	10,000	300 000,000	1 to 30,000	38
4.....	100,000	300,000,000	1 to 3,000	52
5.....	1,000,000	300,000,000	1 to 300	45
6.....	10,000,000	300,000,000	1 to 30	25
7.....	100,000,000	300,000,000	1 to 3	12
8.....	1,000,000,000	300,000,000	3 to 1	22
9.....	10,000,000,000	300,000,000	33 to 1	20
10.....	10,000,000,000	30,000,000	333 to 1	17
11.....	10,000,000,000	3,000,000	3,333 to 1	13
12.....	10,000,000,000	300,000	33,333 to 1	35
13.....	10,000,000,000	30 000	333,333 to 1	30
14.....	10,000,000,000	3,000	3,333,333 to 1	35
15.....	10,000,000,000	300	33,333,333 to 1	39
16.....	10,000,000,000	30	333,333,333 to 1	22
17.....	10,000,000,000	3	3,333,333,333 to 1	21

inoculated with both organisms is somewhat more similar to that produced by the streptococcus alone than to the change due to *B. proteus* alone. The final concentration of hydrogen ions, however, does not reach the high point characteristic of the streptococcus.

It might be expected that the changes produced in any medium by a given symbiotic group would vary in some degree with a variation in the quantitative proportion of the two organisms in the original inoculum. This was found not to be the case, as will be seen in tables 4

⁴ Jour. Bacteriol., 1920, 5, p. 441.

and 5. The technic of the tests of table 4 was to prepare suspensions of the 2 organisms and make a series of dilutions of each. A series of large Smith fermentation tubes of mannite broth were then inoculated with 0.1 c.c. of the original streptococcus suspension, then each of these tubes in turn with 0.1 c.c. of a different dilution of the proteus suspension. Another series of tubes was inoculated with 0.1 c.c. of the original proteus suspension and the same amount of the different dilutions of the streptococcus suspension. To obtain the actual numbers inoculated in each case, 0.1 c.c. of several of the different dilutions of the 2 original suspensions were plated out and the number of each species inoculated into each tube calculated. The tubes were incubated and daily gas readings taken. Copious gas formation took

TABLE 5
INFLUENCE ON GAS PRODUCTION BY A SYMBIOTIC PAIR WHEN ONE ORGANISM IS INCUBATED FOR A SHORT TIME BEFORE INOCULATING THE OTHER

Time of Inoculating Streptococcus N ₁	Time of Inoculating B. proteus L ₂	Time Interval in Hours	% Gas in 10 Days
8:30	8:30	0	30
8:30	9:30	1	21
8:30	10:30	2	20
8:30	11:30	3	18
8:30	12:30	4	20
8:30	1:30	5	16
9:30	8:30	1	24
10:30	8:30	2	20
11:30	8:30	3	27
12:30	8:30	4	18
1:30	8:30	5	26

place in all tubes, but the amount had no relation to the numerical ratio of the 2 organisms in the original inoculum. While there was some variation in the amount of gas formed, this variation was not greater than was found in duplicate or triplicate tubes inoculated with constant amounts of both organisms. The plan of the experiments of table 5 was to inoculate equal numbers of the 2 organisms, but to allow one a short incubation period before inoculating the other. The results were the same as in the experiments just described, but show, in addition, that a slight accumulation of the decomposition products of one organism has no effect on gas production by the symbiotic complex.

Another observation which leads us to assume that gas production in mannite or lactose broth is a quality of this symbiotic pair of organisms quite independent of quantity relations as well as of some other factors, such as temperature, is that when daily transfers were made from one tube (mannite or lactose broth) in which the 2 had been

grown to another tube of the same medium, and from the second, after 24 hour's growth, to a third, and so on, there was no tendency for gas production to diminish or for one organism to disappear from the mixture. These transfers were made over a period of 40 days and gas production following the last transfer was just as vigorous as in the first tube. At room temperature the results were the same except for a slowing down of gas production, due, presumably, to a less rapid multiplication.

The effect of the composition of the medium on gas production was tested especially with regard to the concentration of peptone and the

TABLE 6

THE EFFECT OF VARYING THE CONCENTRATIONS OF PEPTONE ON SYMBIOTIC GAS PRODUCTION FROM MANNITE BY *B. PROTEUS* L₂ AND *STREPTOCOCCUS* N 1

Percentage of Peptone in Medium	% Gas. Average of 5 Tubes								P _H after 18 Days Incubation
	1 Day	2 Days	3 Days	4 Days	5 Days	11 Days	13 Days	18 Days	
0.1	2-5	16	25	27	29	33	34	35	6.3
0.25	2-5	14	25	31	34	43	42	40	6.4
0.5	2-5	6	11	15	18	32	32	..	6.4
1.0	2-5	6	13	16	23	41	41	..	6.4
2.0	2-5	3	10	18	32	52	51	..	6.6

TABLE 7

EFFECT OF INCREASING BUFFER CONTENT OF MEDIUM

Balanced Phosphate Mixture per Liter	% Gas. Average from 4 Tubes						
	1 Day	2 Days	3 Days	4 Days	6 Days	7 Days	12 Days
1 gm.	5	11	16	19	23	25	27
2 gm. ..	5	18	27	27	33	31	32
3 gm.	5	16	22	26	23	23	..
4 gm.	5	18	23	26	29	30	..
5 gm.	5	24	28	29	27	27	..

amount of buffer present. In table 6 are given data showing the influence in the peptone concentration. Table 7 gives the effect of variation in the amount of buffer. The basic medium was the same in each case. It consisted of a broth containing 3 gm. of meat extract per liter and having a hydrogen-ion concentration of P_H 6.8. In the case of the medium of table 6, 4 gm. per liter of a balanced phosphate mixture were added, and the amount of peptone varied as indicated. In the other experiment, 0.2% of peptone was added, and the amount of buffer varied. The gas percentages of table 6 are the averages from 5 different tubes. Those of table 7 are the averages of 4 tubes. It will be seen from these tables that the effect of increasing the peptone is to

slow down gas production, copious gas formation taking place earlier in those tubes containing the least peptone. We seem to have here the reverse of a protein sparing action; that is, carbohydrate metabolism appears to be lessened by an increase in the available nitrogenous constituent of the medium. The effect on the final hydrogen-ion concentration of varying the concentration of peptone was practically nil. Likewise the variation in the quantity of buffer seems to have no influence on the amount of gas produced.

THE PROBABLE EXPLANATION OF SYMBIOTIC GAS PRODUCTION

So far as their ability to attack the carbohydrates, alcohols, glucosides and other fermentable substances is concerned, bacteria are divided into 3 groups, the nonfermenters, the acid formers and the gas formers. In the first class, we place organisms such as *B. fecalis-alkaligenes* and *B. pyocyaneus* which are incapable of breaking down any of the commonly used fermentable materials. In the second group fall such organisms as *B. typhosus*, the streptococci and staphylococci which are capable of fermenting certain of the carbohydrates, alcohols, etc., with acid formation but without the production of gas. The third group comprises a large list of species which break down fermentable compounds always with the production of gas as well as acid. If now we examine the organisms of table 1 with regard to their arrangement into these 3 groups, we find that the first group is not represented at all but that each symbiotic pair consists of an acid former capable of fermenting the sugar used in the experiment and a gas former. A large number of combinations not formed in this way were tested for gas formation, but with negative results. It is understood, of course, that in the tabulated tests only those gas formers were selected which are incapable alone of attacking the sugar used.

Considering these qualities of the gas producing pairs of organisms, a progressive action seems to be clearly indicated leading to the formation of gas in these cases. The degradation of the sugar in question is begun by the acid former and, in the course of this decomposition, substances are formed which are utilizable by the gas former, and gas production results. As to the actual chemistry of the process, we can only make certain deductions.

In the first place, it is evident that the substance attacked by the gas-forming member of the symbiotic pair, that is, the actual mother substance of the gas, is not an end product of the action of the acid former. We repeatedly failed in attempts to produce gas by inocu-

lating a gas-forming organism into a medium on which an acid former had been grown, but which had subsequently been sterilized. Sterilization was accomplished both by filtration and by heat. Gas production never resulted for us except when both organisms were growing simultaneously in the medium. If the proposed theory of gas formation be the true one, we must assume that the mother substance of gas is an intermediary product rather than an end product of the acid fermentation of the sugar, and that this intermediary product, even in pure cultures of the acid former, is decomposed as fast as formed. We have the situation, then, in which one organism of a symbiotic pair gives rise to a substance which both organisms can decompose but with the formation of different end products. In the one case, acids only are the end products; in the other, gas and, presumably, acids also.

This type of symbiotic action differs from the one described by Burri and Stutzer.² In the latter, the reaction takes place in 2 distinct and separable stages. *B. coli* breaks down the nitrate to nitrite, which becomes the mother substance from which nitrogen gas is produced by *B. denitrificans*. The nitrite is an end product of the action of *B. coli*, therefore simultaneous action of the 2 organisms is not necessary to the attainment of the desired end. The symbiotic relationship in this case is a loose one.

Following this reasoning, we can make certain deductions from our experiments as to the chemical nature of the hypothetical compound which we have assumed to be the precursor of gas in our tests. In our first experiments, which were made with lactose and saccharose, we suspected that inversion might be the first step in the decomposition of these sugars and that the monosaccharides thus formed might be the mother substances from which gas was derived. Glucose, especially, was suspected as being the one sugar attacked by all fermenting organisms. When mannite was added to the list, however, and indeed, proved to give gas in greater abundance than any other substance tested, the chemistry seemed too involved to assume a preliminary transformation of this alcohol to glucose. Later results giving symbiotic gas formation from such compounds as fructose, salicin, and, in one or two cases, from glycerol, led us to believe that we must look to a much simpler substance than glucose for our immediate precursor of gas. Frequent chemical tests for glucose in sugar mediums in which an acid former had grown were all negative.

It is probable that the chemical compound which acts as the mother substance of gas in these symbiotic reactions is the same as when the

gas formers are grown in pure culture on a sugar they are capable of fermenting. It was thought that the H_2/CO_2 ratios might throw some light on this question, but this value was found to vary so greatly in repeated tests that no conclusions could be drawn from it. Experiments are now in progress by which it is hoped some information may be gained on this problem through the application of symbiotic phenomena.

It may be suggestive in connection with these observations on the nature of the mother substance of gas to note that König,⁵ Pakes and Jollymann,⁶ Omelianski,⁷ Loew⁸ and Patrouillard⁹ reported the formation by bacteria of CO_2 and H_2 from the salts of the lower fatty acids.

DISCUSSION AND CONCLUSIONS

This paper deals with a new phenomenon, namely, the gaseous fermentation by two organisms growing in symbiosis of a substance from which neither organism acting alone can produce gas. From published observations and many others which it did not seem necessary to more than mention, it is concluded that this phenomenon occurs commonly and that therefore a full understanding of it may be of much practical value. It is possible that this type of gas formation often may give rise to false impressions in the case of the presumptive test for *B. coli* in water and sewage analysis. There are many combinations that could give gas from lactose. The general law governing this phenomenon which is deduced, namely, that a combination of an acid former capable of fermenting the given sugar with a gas former not capable of fermenting this sugar in pure culture may be expected to give gas, should serve to explain in some cases our inability to isolate *B. coli* from lactose tubes showing considerable quantities of gas. It is not unlikely also that many cases of gas formation in canned goods may be due to symbiotic reactions of this kind. Recently an interesting practical example of symbiotic gas formation was encountered. A culture of Morgan's bacillus was received which when tested on the sugars was found to give gas on lactose. It was about to be discarded as *B. coli*, but on more careful examination it was found that it was a contaminated culture of *B. morgani*, the associated organism being a gram-negative bacillus of similar morphology which fermented

⁵ Ber. d. deutsch. chem. Gesellsch., 1881, 1, p. 211.

⁶ Proc. Chem. Soc., 1901, 17, p. 29.

⁷ Centralbl. f. Bakteriolog., 1904, 11, 2, pp. 177, 256 and 317.

⁸ Ibid., 1879, 12, p. 462.

⁹ Compt. rend. Acad. d. sc., 1877, 84, p. 553.

lactose with acid formation but without gas. It appears, then, from these experiments that when inoculation of a mixture of organisms is made into a medium containing a fermentable substance, the appearance of gas in this medium cannot be taken as *prima facie* evidence that an organism is present in the mixture which produces gas on the substance used.

That the qualitative rather than the quantitative composition of a symbiotic group of organisms is likely to be the factor which determines the nature of the chemical changes produced is implied by our results with the complex, *B. proteus-vulgaris* and *Streptococcus fecalis*. It is fully realized, however, that what is true of this pair may not be true of other groups. Nevertheless, we are led to hope that the problems of symbiosis may prove not to be greatly complicated by quantitative relationships.

It is believed, also, that some slight contribution is made by this study to a better understanding of the chemistry of bacterial fermentation by showing that the immediate precursor of gas is a simple substance which is often an intermediary product of the decomposition of sugars by those organisms which produce acid but not gas.

INTESTINAL FLORA IN DIARRHEA

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It is generally recognized that the feeding of grain foods and other carbohydrates, particularly lactose and dextrin, to normal animals favors a predominance of aciduric bacteria in the feces, while the feeding of animal proteins favors the development of proteolytic organisms. Herter and Kendall,¹ in experiments on monkeys and cats, were the first to correlate definitely floral groups with the chemical composition of ingested food, and more recently Torrey,² Rettger and Cheplin,³ Cannon⁴ and others have confirmed their results. Factors other than diet may influence the fecal flora, however, as shown by the recent experiments of Dragstedt, Cannon and Dragstedt,⁵ who found that complete obstruction or stasis of the intestinal contents in dogs resulted in a proteolytic flora irrespective of the character of the diet.

The aciduric flora comprises a group of organisms which have the property of growing in fermentation mediums of a degree of acidity that is incompatible with the development of other bacterial forms. Though the group is not well defined, *B. acidophilus* is most commonly found in the adult and less frequently are encountered strains of *B. coli* that have the faculty of growing in a highly acid medium. In the large intestine of the normal human adult the viable bacteria are chiefly proteolytic in character because of the absence of utilizable carbohydrates and the presence of available protein. Colon bacilli are the predominating bacteria, and although they prefer dextrose, as this under normal conditions is not available, their activities become proteolytic.

In my study of the fecal flora of several patients, in which diarrhea was the chief symptom, I applied methods used by others in the study of the effects of diet on the fecal flora. The proportion of aciduric bacteria to proteolytic ones was determined by the method of Cannon, the so-called colon-acidophilus ratio, which in his experiments on rats

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¹ Jour. Biol. Chem., 1908, 5, p. 293.

² Jour. Med. Research, 1919, 39, p. 15.

³ Intestinal Flora, 1921.

⁴ Jour. Infect. Dis., 1921, 29, p. 369.

⁵ Ibid., 1922, 31, p. 209.

and normal human adults varied with the character of the diet. Particular attention was paid to *B. welchii* because of the different opinions as to its relation to certain types of diarrhea and because of the confusion which exists concerning its proteolytic powers. Herter⁶ considered it a "strictly anaerobic putrefactive organism which attacked proteins vigorously, forming hydrogen, carbon dioxide and perhaps methane gas," while Hall⁷ believes it to be only mildly proteolytic. A complete review of the literature on this phase of the activities of *B. welchii* may be found in Simonds' monograph.⁸

METHODS

As the proteolytic organisms are chiefly gram-negative and the aciduric gram-positive, gram-stained smears of all specimens were studied.

A small sample of feces was weighed accurately in a sterile bottle and sufficient sterile water added to make a dilution of 1:100. The suspension was thoroughly emulsified by shaking vigorously for about 15 minutes. Smears of the 1:100 dilution were stained by the Gram method and examined for the approximate proportion of gram-negative and gram-positive organisms. Quantitative estimations of *B. welchii* were made by the methods of Simonds⁸ in which the 1:100 fecal suspension was diluted serially to 1:51,200 and 1 c.c. of each dilution added to tubes of sterile whole milk, heated for 15 minutes at 80 C., and quickly cooled. Tubes showing stormy fermentation after 72 hours' incubation, with an odor of butyric acid, and the presence of gram-positive bacilli in smears of the culture were called positive; 25 c.c. of the 1:100 dilution were placed in an evaporating dish, previously weighed, and evaporated to dryness over a water bath and then desiccated in an Arnold sterilizer; by weighing the evaporating dish again the weight of the total solids in 25 c.c. of 1:100 dilution could be computed, and considering that 1 c.c. of the highest dilution, which caused stormy fermentation, contained at least one spore of *B. welchii*, the minimum number of spores in a gram of dried feces was computed accordingly. An attempt was made to utilize the method of Cannon for determining the proportion of aciduric to proteolytic organisms,

⁶ Jour. Biol. Chem., 1906-1907, 2, p. 1.

⁷ Jour. Infect. Dis., 1922, 30, p. 445.

⁸ Monographs of the Rockefeller Institute for Medical Research, 1915, No. 5.

the so-called colon-acidophilus ratio. This method consists of inoculating plates of Torrey's⁹ beef liver glucose agar (5% acid) and Ayers-Rupp¹⁰ agar with equal quantities of the same dilution of a fecal suspension. The proportion of deep red metallic colonies in the Ayers-Rupp medium to the small fluffy colonies in Torrey's medium, after 48 hours' incubation, is the colon-acidophilus ratio. The method was first tried with fecal suspensions from rats fed only milk, bread and lactose and from another group fed a diet limited to meat. As in Cannon's experiments, the proportion of acidophilus-like organisms to colon bacilli was greatly increased in the rats on the lactose diet. The method was not entirely successful, however, in studying the flora of

TABLE 1
NORMAL STOOLS

Cases	Smears		Type of Flora	B. welchii Spores		
	Percentage Gram-negative	Percentage Gram-positive		Weight of Dried Residue of 25 c.c. 1:100 Dilution of Feces	Highest Dilution Causing Stormy Fermentation	Spores per Gram of Dried Feces
1	99	1	Proteolytic	0.050	100	500
2	95	5	Proteolytic	0.036	0	0
3	99	1	Proteolytic	0.060	800	3,500
4	90	10	Proteolytic	0.055	100	500
5	96	4	Proteolytic	0.040	400	2,500
6	95	5	Proteolytic	0.072	1,000	6,000
7	85	15	Proteolytic	0.068	100	400
8	99	1	Proteolytic	0.042	200	1,200
9	99	1	Proteolytic	0.050	200	1,000
10	96	4	Proteolytic	0.042	800	4,800

human stools, because in two cases the smears were definitely gram-positive while the colon acidophilus ratio did not differ from that found in stools known to be proteolytic. The method was used in all cases, and usually more organisms grew on the highly acid medium when the smears had a gram-positive tendency. No attempt is made in the tables to give a ratio in numerical terms, but when the highly acid medium contained the greater number of colonies it is called aciduric.

RESULTS

As shown in table 1, the fecal flora of all normal stools was proteolytic in type, the smears showed a preponderance of gram-negative bacteria, and spores of *B. welchii* were present in 9 of 10 cases.

⁹ Jour. Bacteriol., 1917, 2, p. 435.

¹⁰ Ibid., 1918, 3, p. 433

In table 2 are recorded the results in 8 patients who had a diarrhea as the result of intestinal lesions, ulcerative or otherwise. The findings in all are strikingly similar, smears and flora differing in no way from those of normal stools, but with a definite increase of *B. welchii* spores.

TABLE 2
DIARRHEA WITH INTESTINAL LESIONS

Cases	Smears		Type of Flora	B. welchii Spores		
	Per-centage Gram-negative	Per-centage Gram-positive		Weight of Dried Residue of 25 c c. 1:100 Dilution of Feces	Highest Dilution Causing Stormy Fermentation	Spores per Gram of Dried Feces
10. Polypoid colitis; man 43; severe attacks of diarrhea for 10 years, lasting 2 to 3 months; loss in weight, anemic; on soft carbohydrate diet; stools semifluid, foamy, 10 to 12 daily; blood, once only; flatulence; antifermentative diet made him worse; polypoid lesions of entire colon postmortem	99	1	Proteolytic	0.015	51,200	850,000
11. Ulcerative colitis; man 31; severe diarrhea 3 months; stools fluid, blood, pus, mucosal shreds present; no improvement on carbohydrate diet	80	20	Proteolytic	0.010	25,600	640,000
12. Amebic dysentery; sailor 28; duration 3 years, 4 or 5 attacks; stools liquid, 20 to 30 daily, with amebae, pus and blood	90	10	Proteolytic	0.008	6,400	200,000
13. Tuberculous enteritis; woman, 33; stools contain blood, pus, tubercle bacilli..	90	10	Proteolytic	0.016	1,600	25,000
14. Tuberculous enteritis; woman, 29	99	1	Proteolytic	0.021	51,200	604,000
15. Tuberculous enteritis; woman, 48	60	40	Proteolytic	0.030	400	3,500
16. Tuberculous enteritis; woman, 30	95	5	Proteolytic	0.020	25,600	320,000
17. Carcinoma of colon with diarrhea for 6 months.....	95	5	Proteolytic	0.015	25,600	425,000

Streptococci were relatively abundant in the smears of case 16, a case of tuberculous enteritis.

Cases which did not have any pus or blood in the feces or any other evidence of intestinal lesions are grouped in table 3. The diagnoses in this group are for the most part incomplete. Cases 1, 2

TABLE 3
DIARRHEA WITHOUT INTESTINAL LESIONS

Cases	Smears		Type of Flora	B. welchii Spores		
	Per-centage Gram-negative	Per-centage Gram-positive		Weight of Dried Residue of 25 c. c. 1:100 Dilution of Feces	Highest Dilution Causing Stormy Fermentation	Spores per Gram of Dried Feces
1. Fermentative diarrhea; man, 26; mild diarrhea irregularly for 2 years, usually after eating sweets and easily stopped by limiting sweets; stools foamy, sour, no blood or pus, mucus occasionally...	60	40	Aciduric	0.033	0	0
2. Fermentative diarrhea; man, 25; frequent attacks for 5 years, recently severe; flatulence; stools copious, foul, gas riddled; antifermentative diet of no avail; cause obscure	95	5	Proteolytic	0.030	25,600	220,000
3. Acute diarrhea; young woman; single attack in August, duration 5 days; stools watery, checked by boiled milk diet	99	1	Proteolytic	0.020	12,800	160,000
4. Man, 50; periodic diarrhea every 6 months, duration 2 to 3 days; stools foul, watery, 10 to 12 daily; cause obscure	96	4	Proteolytic	0.018	6,400	90,000
5. Acute diarrhea; boy, 15; fever 101; toxic; checked by fasting	95	5	Proteolytic	0.028	25,600	240,000
6. Cathartic colitis; woman, 25; alternating constipation and diarrhea for 8 years; colon spastic; stools soft and mushy	90	10	Proteolytic	0.032	12,800	108,000
7. Acute diarrhea, woman, 40, with pulmonary tuberculosis; watery stools for one day after eating fruits.....	50	50	Aciduric	—	0	0
8. Man, 35; diarrhea, fever for 3 weeks; stools liquid, 10 to 15 daily, observed only once; obscure	99	1	Proteolytic	0.018	51,200	720,000
9. Fermentative diarrhea; man, 45, obese, heavy eater, fond of sweets; always has 2 to 3 mushy stools daily; flatulence; abdominal distress; colon tender; stools well formed when eating moderately	90	10	Aciduric	0.025	100	1,000

and 9 were diagnosed as fermentative diarrhea because of the foamy, gas-riddled appearance and sour odor of the stools, positive Schmidt fermentation tests within 12 hours and rather definite history of carbohydrate foods as the causative factor. The flora in cases 1 and 9 was aciduric, but in case 2 it was definitely proteolytic. Spores of *B. welchii* were absent in case 1, reduced to a small number in case 9, but in case 2 large numbers were present. Here are two cases of fermentative diarrhea that have an aciduric flora, while one case, also fermentative, had a proteolytic flora. It was thought that perhaps the process might be alternately one of putrefaction and fermentation; but in cases 1 and 2, which were under observation for a considerable period of time, there was no change in the type of flora during different attacks of diarrhea. Case 2 previously had been diagnosed as "gas bacillus diarrhea," but treatment with a buttermilk and protein diet aggravated rather than relieved it. Although case 7 presented none of the clinical features of a fermentative diarrhea, the flora was aciduric and spores of *B. welchii* were absent. The flora of the other cases of this group was similar to the flora in the diarrheas due to intestinal lesions, and of course there is no proof that lesions were not present in these cases.

The presence of large numbers of spores of *B. welchii* in proteolytic diarrheal stools is evident from the results. As the stools from two patients who had taken a cathartic showed a similar increase in spores, the increase might be attributed to the rapid passage of the intestinal contents. It would not account, however, for the results obtained with the dry feces of rats that were fed meat, for in the preliminary experiments the stools of rats on a high protein diet showed a marked increase of *B. welchii* spores. The absence of *B. welchii* spores in cases 1 and 9, whose flora was aciduric, is also significant. Cannon,⁴ in his studies on normal persons and rats fed alternately mammalian tissue and a diet rich in lactose, also observed large numbers of *B. welchii* in the flora of stools that were predominantly proteolytic and a decreased number when the stools were aciduric. These results can be explained by the ability of *B. welchii* to exist as a sporulating or nonsporulating form, dependent on the character of the medium. Thus Simonds⁸ has shown that a sporulating culture inoculated into a medium containing sugar immediately ceases to germinate, and further that when a sugar-free medium is inoculated with a nonsporulating culture, spores form sooner or later.

As *B. welchii* spores are present in great numbers in a proteolytic flora and absent in a markedly aciduric flora, it seems likely that the spores are absent when there is sufficient carbohydrate to allow all to germinate and are increased in proportion to the insufficiency of carbohydrate. It also seems likely that the stormy fermentation test for *B. welchii* spores might be used in the clinical study of the intestinal flora.

SUMMARY

The intestinal flora in 8 cases of diarrhea, connected with intestinal lesions, was proteolytic, while in 2 cases of fermentative diarrhea the flora was aciduric; but in another similar case, the flora was proteolytic.

Spores of *B. welchii* were present in great numbers in stools with a proteolytic flora and absent in those with an aciduric flora.

VARIATIONS IN STREPTOCOCCUS HEMOLYTICUS ON ANIMAL PASSAGE

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It has been recognized for a number of years that a micro-organism, under certain conditions, may produce colonies differing greatly from the type of colony considered characteristic of that organism. Variations in the type of colony formed by the typhoid bacillus have been described by von Lingelsheim,¹ and similar variations for other bacteria have been reported by others. There have been few instances, however, in which these variations in the mode of growth have been successfully correlated with variations in virulence. Thjötta and Eide,² for instance, isolated from the urine of a carrier a paratyphoid bacillus producing large mucoid colonies, which was no more virulent for mice than the ordinary type of paratyphoid bacilli isolated from the same individual. The work of DeKruif³ has been elucidative in this respect, and he has shown conclusively that the bacillus of rabbit septicemia could be separated into two types, differing from each other in the kind of colony produced and in the mode of growth in broth. He showed, furthermore, that one of these two types was more virulent for animals than the other, and that one type could arise from the other.

Recent work of Cowan⁴ has demonstrated that the virulence of hemolytic streptococci also may vary with variations in the type of colony and the character of the growth in broth. By careful selection of the extremes of the 2 types of growth over a period of 4 months, she was able to differentiate each strain into 2 types, "smooth" and "rough," that remained true to form on subsequent transfers. The "smooth" type produced regular translucent colonies on agar and grew as an even cloud in broth. The "rough" type produced rather granular opaque colonies with irregular borders, and in broth grew as a sediment in the bottom of the tube, leaving a clear supernatant fluid. The "smooth" type was definitely more virulent for mice and rabbits than the "rough" type. There was no demonstrable difference in the hemolytic activity of the 2 strains.

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¹ *Centralbl. f. Bakteriol., I. O.*, 1913, 68, p. 577.

² *Jour. Bacteriol.*, 1920, 5, p. 501.

³ *Jour. Exper. Med.*, 1921, 33, p. 773; 1922, 35, p. 561 and 36, p. 309.

⁴ *Brit. Jour. Exper. Path.*, 1922, 3, p. 187.

A special type of colony produced by some strains of hemolytic streptococci on blood agar has been described previously in connection with epidemics of "septic sore throat," due as a rule to a contaminated milk supply. These colonies are large and moist, varying in diameter from 1 to 8 mm. They have sometimes been spoken of as "dew-drop colonies," on account of their raised and transparent appearance. In broth, the organisms grow as a diffuse cloud and produce only short chains. Some authors describe them as encapsulated, while others do not. These organisms are referred to as *Streptococcus epidemicus*, though it is recognized that all strains may not be the same.

Streptococci with these general characteristics were described by Davis and Rosenow,⁵ who isolated them from an epidemic of sore throat in Chicago spread by milk. Similar organisms were obtained from the same sort of epidemic by Hamburger,⁶ in Baltimore. Since then, the epidemiology and bacteriology of the affection have been investigated particularly by Smith and Brown⁷ and by Brown and Orcutt.⁸ Keegan⁹ has described an epidemic that was prosodemic in character.

The first thorough bacteriologic study of streptococci of this type was made by Davis,¹⁰ who recorded some very interesting observations on the lack of permanence of the special characteristics, and suspected the relationship of these special characteristics to virulence. These observations of Davis have been confirmed, at least in part, by the work reported in this paper, and appear to be of an ever increasing importance at this time, when variation phenomena in the cultural characteristics of bacteria, accompanying changes in virulence, are being more generally looked for. Davis emphasized the occurrence of a capsule, though he found that the profuse moist appearance of the colonies and the capsule went hand in hand. He was of the opinion that the organisms are modified forms of *Streptococcus pyogenes*, and demonstrated that an organism having lost its capsule on artificial medium, regained it on animal passage. He also isolated a hemolytic streptococcus from the udder of a cow that was suspected of being concerned in the origin of the epidemic which he studied, and a similar organism from the

⁵ Jour. Am. Med. Assn., 1912, 58, p. 773.

⁶ Ibid., 1912, 58, p. 1109.

⁷ Jour. Med. Research, 1914-15, 31, p. 455.

⁸ Jour. Exper. Med., 1920, 31, p. 49.

⁹ Jour. Am. Med. Assn., 1919, 72, p. 1434.

¹⁰ Ibid., 1912, 58, p. 1852.

throat of a girl on the same farm. These organisms, on successive animal passage, both acquired a capsule and the other characteristics of *Streptococcus epidemicus*.

THE PRESENT WORK

The basis of my own observations was an organism obtained during the course of a throat culture survey of army recruits at Fort Slocum, N. Y.¹¹ The hemolytic streptococci, isolated during this survey, were transferred to broth, from which pour plates were made. Single colonies were fished to blood broth and, after 24 hours' growth, placed in the icebox. All blood used had been incubated for 48 hours to test its sterility. Transfers were made to new blood broth from every 4 to 6 weeks.

In Feb., 1921, 5 c.c. of a 24-hour broth culture of one of the strains, 357A, were injected into the right pleural cavity of a rabbit for the purpose of obtaining a purulent empyema fluid for classroom work. This strain was selected quite at random. It produced the beta type of hemolysis of Smith and Brown, with a hemolytic zone of 2.2 mm., and 24-hour broth cultures produced hemolysis when mixed with an equal quantity of a 5% suspension of rabbit red blood cells and placed in the water bath at 37 C. for 2 hours. The fermentation reactions were those of *Streptococcus pyogenes* in Holman's classification. The strain had been obtained from a person whose tonsils were described as slightly hypertrophied. Two throat cultures had been made of this recruit, only one of which was positive for hemolytic streptococci.

The rabbit receiving the injection died 40 hours later. There was a fibrinous deposit on the surface of the pleurae and each pleural cavity contained about 5 c.c. of a cloudy, slightly bloody fluid. On smear, there were many leukocytes and a large number of gram-positive cocci.

Two types of colonies developed from cultures made from the pleural fluid on blood-agar plates. The great majority of the colonies were like the ordinary colony of *Streptococcus hemolyticus*, that is, about 0.8 mm. in diameter, with slightly darkened centers, and fragmenting, as a rule, when touched with a platinum wire. These colonies were exactly the same as those produced by the strain 357A, and, for want of a better term, are subsequently referred to as "normal" colonies. They could hardly be described as rough. The other type of colony was of a striking appearance, although the proportion of them was

¹¹ Military Surgeon, 1921, 48, p. 561.

only about 1:500 of the "normal" type, and of relatively enormous size, varying from 6 to 8 mm. in diameter. They were almost entirely clear and transparent, their surfaces were smooth and convex, due to their elevation, and the edges regular. A platinum wire penetrated without resistance into their substance, and on withdrawing the wire, the colony was seen to have a slightly sticky character, though this was not marked. The colonies rapidly dried to thin films, with the concentric rings noted by Keegan.

Stains for capsules from the colonies, and later from the animal body, were consistently negative. The Hiss capsule stain was used. India ink, stated by Brown and Orcutt to best demonstrate the capsules of *Streptococcus epidemicus*, was not tried. It was rather surprising that an organism producing as mucoid a colony as this did not have an easily demonstrable capsule. In all probability, the substance about the organisms was so dilute, as shown by the watery consistence of the colonies and the rapid drying, that it retained the stain only with great difficulty.

These large colonies will be described later simply as "moist." They were hemolytic, though the hemolytic zone did not extend far beyond the borders of the colony. When the colony was scraped away the underlying medium was seen to be of a particularly clear appearance. A few colonies were near enough to each other to become confluent.

On transfer to broth, the "normal" type grew as a sediment in the bottom of the tube, with clear supernatant fluid. The "moist" type grew as a diffuse cloud, with little tendency to settle out even after standing for several days on the desk. Stains made from broth showed the "moist" type as short chains. The "normal" type grew as much longer irregular chains, often entangled with each other. The fermentation reactions of the two types were the same as of the parent strain. Neither was bile soluble.

COMPARATIVE VIRULENCE OF THE "MOIST" AND "NORMAL" TYPES

At the suggestion of Major H. J. Nichols, the virulence of the 2 types was tested in animals. A rabbit was injected intravenously with 1 c.c. of a 24-hour broth culture of the "moist" type, and another rabbit received a similar quantity of the "normal" type. The rabbit receiving the "moist" strain was found dead the following morning; there were no lesions except small hemorrhagic areas in the lungs. Smears from the heart blood showed occasional cocci, and culture of the heart's blood on blood-agar plates showed a pure culture of numerous

colonies of the "moist" type, confluent as a rule, though isolated colonies were of the same size as those originally seen. The rabbit receiving the "normal" type showed no symptoms, and was discarded 10 days later.

Two additional rabbits were similarly injected with 24-hour broth cultures of the 2 strains, except that this time 2 c.c. of the "normal" type were given and only 0.25 c.c. of the "moist" type. Blood cultures from both animals on the following day were positive, and, on being plated out, each yielded only colonies of the type injected. The "moist" colonies from the blood culture were still from 6 to 8 mm. in diameter. The animal receiving the "moist" strain died 72 hours after injection, and "moist" colonies were obtained in pure culture from the heart's blood. The animal receiving 2 c.c. of the "normal" strain presented no symptoms, and was discarded 10 days later.

TABLE 1
COMPARATIVE VIRULENCE FOR MICE OF 1 C.C. OF SUCCESSIVE DILUTIONS OF BROTH CULTURES

	Dilution				
	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
"Moist".....	Died in 16 hours	Died in 16 hours	Died in 16 hours	Died in 24 hours	Died in 24 hours
"Normal".....	Died in 36 hours	Died in 48 hours	Died in 48 hours	Survived	Survived
357A.....	Died in 16 hours	Died in 48 hours	Survived	Survived	Survived

The virulence of the 2 types was also tested with mice, and in addition the virulence of the original strain, 357A, which had not been passed through an animal. As mentioned, there was no difference in the cultural characteristics of the "normal" type and 357A. The results are shown in table 1.

Hemolytic streptococci were isolated from the hearts blood of all mice dying. In each case the type injected was recovered.

HEMOLYTIC ACTIVITY OF THE TWO STRAINS

In pour plates the "normal" strain produced the same type of hemolysis as the parent strain, 357A, that is, a clear zone of approximately 2.2 mm. in diameter. The outline of the colony was sometimes "complex." Deep colonies of the "moist" strain produced the same type of hemolysis except that the hemolytic zone was slightly larger.

The colonies were never "complex." The special type of colony produced by the organism would not have been suspected from the examination of deep colonies, though in pour plates occasional colonies developed on or near the surface, in which case the typical "moist" appearance was always assumed. Streak plate made of the two strains on human blood showed the "moist" strain to be definitely more hemolytic than the "normal" strain. Unfortunately, pour plates were not made with human blood.

The hemolytic activity of the 2 strains was titrated with a 5% suspension of washed rabbit cells, using 24-hour broth cultures of the organisms. Varying amounts of the broth cultures were set up against 0.5 c.c. of the cell suspension. The tests were read at the end of 2 hours at 37 C. in the water bath (table 2). The whole broth culture

TABLE 2
TITRATION OF THE HEMOLYTIC ACTIVITY OF BROTH CULTURES

	Amount of Broth Culture Used							
	0.025	0.05	0.10	0.20	0.30	0.40	0.50	0.60
"Moist".....	+++	+++	++++	++++	++++	++++	++++	++++
"Normal".....	—	—	—	±	+	+	+++	+++
357A.....	—	—	±	±	+	++	+++	+++

++++ indicates complete hemolysis of the cells, and — no hemolysis. The other symbols represent intermediate stages of hemolysis.

was used, after being shaken up. The greater hemolytic activity of the "moist" strain is marked and striking, despite the fact that on surface streaks the hemolysis did not seem great.

GRADUAL LOSS OF SPECIAL FEATURES OF MOIST STRAIN

Daily transfers of the "moist" strain had been made on blood-agar plates. In each instance the largest and most mucoid colony on the plate was selected for transfer. The virulence and hemolytic tests noted above were all made from broth transfers obtained from not later than the sixth generation after isolation from the original rabbit. The marked moist and mucoid character of the colonies began to diminish on successive transfers almost from the beginning, especially as exemplified by the diameter of the colony. By the seventh generation the "moist" type was growing as colonies only 3 mm. in diameter, though the moist character of the colony was still present and marked. Daily transfers were similarly being made of the "normal" type, each

time a single colony also being selected for transfer. Occasional colonies of the "normal" type seemed to have a tendency to become "moist," appearing slightly more transparent and of greater diameter than the other colonies of the "normal" strain. Transfers from these colonies invariably gave rise only to typical "normal" colonies.

After the eighth generation, the plates of the "moist" strain began to show a rather large number of colonies that appeared to be on the borderline between the typical "moist" colony and the typical "normal" colony. These borderline colonies increased on subsequent transfers. In the thirteenth generation it was noted that the only difference in the appearance of the colonies of the 2 strains was that the "moist" strain produced colonies that were slightly larger than the "normal" strain. In the subsequent generation, even this difference had disappeared, and the 2 strains grew exactly alike. Transfers to broth from this generation grew as a sediment in the bottom of the tube with a clear supernatant fluid. The virulence of the formerly "moist" strain was again tested against mice, using 1 c.c. of dilutions from 1:10,000 to 1:1,000,000. None of these mice died, in marked contrast to the first virulence test when the 1:1,000,000 dilution had killed the mouse in 24 hours. Hence the "moist" strain had lost its marked virulence, along with the loss of its other special features.

In this work fresh blood-agar plates, which had been incubated for sterility but which had not begun to dry out, were used. It seemed obvious that a colony of the "moist" type could not develop except in the presence of a sufficient amount of moisture. However, the presence of excessive amounts of moisture, or even the placing of many drops of broth on the surface of the plate and inoculating them with the "moist" strain, had no influence in preventing the disappearance of the special type of colony. The blood-agar plates were made by adding 1 c.c. of defibrinated rabbit blood to 16 to 18 c.c. of beef extract agar, P_H 7.6.

ATTEMPTS TO REPEAT THE ORIGINAL EXPERIMENT

Since the "normal" strain had been transferred daily for 12 days, and each time a single colony had been picked for transfer, thus assuring with a fair degree of certainty a single cell strain, the attempt was made to convert it into the "moist" type by animal passage. A rabbit was injected intrapleurally with 6 c.c. of a 24-hour broth culture. Only "normal" colonies developed from a pleural puncture made 24 hours later. The rabbit died 48 hours after injection. Cultures from

the pleural fluid showed only the development of "normal" colonies. About 10% of the colonies from the heart blood were "moist"; they were of rather small size, however, as the diameter did not exceed 1.5 mm. Transfers from these "moist" colonies gave rise only to "normal" colonies. There were also many colonies on the plates from the heart blood of the rabbit that represented intermediate stages between "moist" and "normal" colonies.

Similar efforts were made to repeat the original observations with the culture 357A. This strain was transferred to broth, incubated for 24 hours, and pour plates made. Single colonies were again transferred to broth, and again replated after 24 hours' growth. This process was repeated four times, after which 5 c.c. of a 24-hour broth culture were injected into the pleural cavity of a rabbit. A pleural puncture was made 48 hours after the injection, and from the fluid obtained a small proportion of "moist" colonies developed, the largest of which were 2 mm. in diameter. These "moist" colonies remained true to form for 2 successive generations, after which subsequent transfers gave rise only to "normal" colonies. This rabbit did not die, and was discarded 10 days later.

Another rabbit was injected intrapleurally with 8 c.c. of a 24-hour broth culture of 357A. On the following day the pleural fluid was cultured. A few "moist" colonies developed on the plates, the largest being 1.5 mm. in diameter. The plates also showed all gradations between the 2 types of colonies. Transfers from the "moist" colonies gave rise only to "normal" colonies. The animal was found dead 40 hours after injection. Cultures from the pleural fluid and from the heart's blood gave rise to a small proportion of "moist" colonies, the moist character of which was more marked than in any experiment since that performed on the original rabbit. They were 2.5 mm. in diameter. Successive transfers from these colonies remained true to form for 3 generations, after which only "normal" colonies developed.

On nearly all the plates from animals, colonies were noted that were intermediate in character between the 2 types. During the course of the work, all stages were observed between the large "moist" colony 8 mm. in diameter, seen on the plate from the first rabbit, and the ordinary "normal" colony. There was no sharp line of demarcation between the 2 types. It had been hoped to obtain again the large "moist" colony from 6 to 8 mm. in diameter, which retained its special features at least long enough for its virulence and other properties to

be studied. Although this was not successfully attained, it is felt that the "moist" colonies of smaller diameter which did develop on the later attempts present strong evidence that the original large colonies were variation forms of the strain injected.

DISCUSSION

It is interesting to note that the original strain, 357A, was obtained from a throat which was practically normal, the only note at the time of culture being that the tonsils were slightly hypertrophied, as determined by gross examination. This fact is perhaps of importance in connection with the epidemiology of "septic sore throat," as it probably means that under conditions not fully understood the ordinary type of *Streptococcus pyogenes* may take on these features, with resulting epidemics, in case of contaminated milk supply, or from the infection of cows with such an organism from human sources, as evidenced by the work of Smith and Brown and of Brown and Orcutt.

It would, however, probably be incorrect to assume at the present time that all strains of hemolytic streptococci may be converted into this special type. Such variation phenomena have not been described by Gay and his associates¹² in connection with the repeated passage of a strain through the pleural cavities of rabbits, though the strain increased markedly in virulence, and became more hemolytic¹³ than the original strain. It is also possible that 357A was at the beginning different from other hemolytic streptococci, as evidenced by its rather marked virulence shown in table 1, where 1 c.c. of a 1:1,000 dilution of a broth culture killed a mouse in 48 hours. Five other "carrier" strains were similarly tested against mice. None of them killed mice in a 1:100 dilution, though 4 of them caused death in a 1:10 dilution.

It is also interesting to note, in this connection, the recent work of Arkwright,¹⁴ who found that marked differences exist in regard to agglutination between strains of the Shiga bacillus arising from colonies of different types. These observations were extended to include members of the paratyphoid B (*Salmonella*) group, by Schuetze,¹⁵ who found that the substrains may differ so much from the parent strain that by ordinary criteria one would be almost justified in regarding them as new and unrelated types. Whether the difficulties in regard

¹² Jour. Infect. Dis., 1921, 29, p. 217.

¹³ Cook, M. W.; Mix, V., and Culvyhouse, E. D.: Jour. Infect. Dis., 1921, 28, p. 93.

¹⁴ Jour. Path. & Bacteriol., 1921, 24, p. 36.

¹⁵ Jour. Hygiene, 1921, 20, p. 330.

to the classification of hemolytic streptococci by the reaction of agglutination are in any way related to these observations of Arkwright and of Schuetze for intestinal bacteria, it is of course difficult to say, though it seems apparent that the agglutination reactions of a strain, growing as the "moist" type described above, could be read with greater ease and certainty than in the case of ordinary strains.

CONCLUSIONS

Evidence is presented that tends to show that the "moist" type of colony, characteristic of the so-called *Streptococcus epidemicus*, is but a temporary character, and may be acquired by an ordinary *Streptococcus hemolyticus*.

The virulence of a strain having acquired this "moist" type of growth is greatly increased, and the virulence decreases with the loss of these special characteristics.

The confirmation of the fact that an ordinary strain of *Streptococcus hemolyticus* may acquire the properties of *Streptococcus epidemicus* is believed to have an important bearing on the epidemiology of "septic sore throat."

It is suggested that, analogous to observations on other bacteria, the antigenic properties of hemolytic streptococci may possibly also vary along with these variations in the character of the colony.

THE INCIDENCE AND CLASSIFICATION OF
STAPHYLOCOCCI IN THE THROATS
OF NORMAL PERSONS AND
OF PERSONS WITH
COMMON COLDS

INFLUENZA STUDIES. XII.*

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It is not necessary to recapitulate here the existing theories concerning the cause of colds. The literature on this subject has been reviewed recently by Bloomfield¹ and by Mudd, Grant, and Goldman.²

Staphylococci should be considered in any systematic survey of the organisms of the upper respiratory tract. They are recognized as common inhabitants of this region, as in fact of all exposed surfaces and most cavities connected with the exterior. Williams, Nevin, and Gurley³ found *Staph. albus* in the nasopharynx of 12 of 75 persons. Bloomfield⁴ showed that *Staph. aureus* and *Staph. albus* are transient in different parts of the air passages and that they are removed mainly by mechanical means. Mudd, Goldman, and Grant⁵ reported that in 4 subjects studied, chilling of the body surface caused an increase in numbers of bacteria already present in the upper respiratory tract, but that *Staph. aureus* and *albus* isolated from the nose under normal conditions were not altered in numbers by this exposure. *Staph. albus* and less commonly *Staph. aureus* are normal inhabitants of the nasopharynx, according to Mackey.⁶

Staphylococci are known to be capable of setting up disease processes of the upper respiratory tract and sinuses varying in degree from a slight inflammation of the mucous membrane to a true pneumonia. Floyd⁷ associated staphylococci with rhinitis in particular and included this organism in vaccines for acute respiratory infections. Mackey⁸ believed that in bronchitis the same variety of organism occurs in the nasal passages and sputum, and that when *Staph. aureus* or *Staph. albus* predominates in the nasal passages it should be used in a univalent or polyvalent vaccine in the treatment of the bronchitis.

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

¹ Bull. Johns Hopkins Hosp., 1921, 32, p. 121.

² Jour. Lab. & Clin. Med., 1921, 6, pp. 175 and 322.

³ Jour. Immunol., 1921, 6, p. 5.

⁴ Bull. Johns Hopkins Hosp., 1921, 32, p. 290; 1920, 31, p. 14. Amer. Rev. Tubercul., 1920, 4, p. 247.

⁵ Jour. Exper. Med., 1920, 32, p. 87.

⁶ Brit. Med. Jour., 1919, 2, p. 159.

⁷ Bost. Med. & Surg. Jour., 1920, 182, p. 389.

⁸ Brit. Med. Jour., 1922, 2, p. 715.

Gardner⁹ reported finding *Staph. aureus* in the upper respiratory tract of three children having colds with gastro-intestinal symptoms. The organism he isolated was able to reproduce in rabbits features seen in the human cases.

Staphylococci may conceivably occur in this region either as potential pathogens or as pathogenic strains transmitted from one person to another. The former supposition seems more likely because of their occurrence in normal conditions and because a lowering of the resisting powers of the host by the primary virus is probably conducive to bacterial invasion by this organism as by others. This secondary invasion was shown by Patrick,¹⁰ who found *Staph. aureus* associated secondarily with influenza and isolated it from the sputum of influenza patients. Mathers¹¹ recovered staphylococcus from the nasal discharges and sputum in 50 of 61 cases of the grip. The staphylococcus is classed with the opportunist bacteria by Davis,¹² who pointed out that certain bacteria normally present in the upper respiratory tract may become active in infections of this region when the resistance of the host is lowered by the original virus. Staphylococci he maintains are of less importance, however, than streptococci or pneumococci and are not known to be capable of initiating respiratory infections of epidemic magnitude. Very much the same opinion is expressed by Bloomfield.¹

A review of the literature on the rôle that the staphylococcus plays in acute conditions of the upper respiratory tract shows that this organism is commonly but not always present in this region in normal persons; that it is rarely capable of producing uncomplicated respiratory conditions; and that it is probably an opportunist, becoming active as a complicating factor or secondary invader.

In this study of the relation of the staphylococcus to common colds, the nasopharynx and occasionally the throat of normal persons and of those suffering with common colds were swabbed with a bent wire swab. Whole blood agar plates were immediately inoculated and incubated at 37 C. Colonies suspected of being staphylococcus were stained by Gram's method, and those giving the typical morphology and arrangement were transferred to plain agar slants. The strains isolated were found to be gram-positive in most instances, but a few were decolorized by this method. An attempt was made to avoid picking duplicate colonies from the same plate, judging them by the size, color, reaction on the blood, and general appearance of the colony. From the agar slants the strains were again streaked on blood agar, hemolysis noted, and single colonies transferred to starch agar slants for stock cultures. They were periodically transferred to other slants of the same medium and stained to be certain that the cultures carried were pure.

⁹ Amer. Jour. Med. Sc., 1918, 155, p. 380.

¹⁰ Lancet, 1919, 196, p. 137.

¹¹ Jour. Amer. Med. Assn., 1917, 68, p. 678.

¹² Ibid., 1920, 75, p. 792.

The method of determining the relation of the staphylococcus to common colds was to compare the incidence and cultural relations of this organism in such conditions and in normal persons. The following tests were made: chromogenesis, hemolysis, gelatin liquefaction, reaction in litmus milk, reactions in solid medium containing dextrose, maltose, lactose, mannite, and glycerol, production of indol, and reduction of nitrate to nitrite. Twenty-four-hour cultures were used in every case.

In all, 223 persons were examined, 143, or 64%, giving positive results for staphylococcus and the remainder, or 36%, being negative; 98 of these persons had colds and 125 were normal. According to their own statements, a few of the normal persons had a slight irritation of the throat at the time of examination. In the normal group, 65, or 52%, were positive for the staphylococcus, while in the group with colds 78, or 80%, yielded positive cultures. It is thus evident that the incidence of this organism is greater in the nasopharynx and throat of persons with colds than in those of normal persons. These results agree roughly with those of Williams, Nevin, and Gurley,³ who found staphylococci in the nasopharynx of 16% of normal persons and 84% of persons with colds.

Seventy strains were isolated from the 65 persons positive for this organism in the normal group and 192 strains from the 78 persons positive in the group with colds. Chromogenesis was determined by prolonged culture on plain and starch agar. Pigmentation was more intense, however, after short periods of culture on mediums containing the simpler carbohydrates. Of the 70 strains from normal persons, 28 (40%) were *Staph. aureus*, 40 (57%) were *Staph. albus*, 1 was *Staph. citreus* and 1 was nonchromogenic. Of the 192 strains from the group with colds, 34 (18%) were *Staph. aureus*, 146 (76%) were *Staph. albus*, 5 were *Staph. citreus*, and 7 were nonchromogenic. Colorless staphylococci have not elsewhere been noted, but they are probably unimportant variants in chromogenesis. They were close to *Staph. citreus* in their reactions and for the sake of simplicity are grouped with that division.

A comparison of the incidence of the chromogenic groups in the two conditions shows that *Staph. aureus* is found in the normal, in even a higher proportion than in the group with colds. This is contrary to the usual published findings. Apparently *Staph. aureus* is just as likely to be an unimportant semiparasite as *Staph. albus*, but it may of course be more potential as a pathogen when once it gets a foothold.

Hemolysis was determined by streaking out the cultures on whole blood-agar plates of uniform thickness. Reactions were noted after 24, 48, and 72 hour periods; 63% of all strains were positive at the end of the 72-hour period. The cocci from normal throats were more actively hemolytic (73% positive) than those from colds (59% positive). Hemolysis has often been suggested as a means of classifying staphylococci, especially when correlated with pathogenicity, but the differences noted here were not significant.

Gelatin liquefaction was studied by inoculating gelatin with cultures of the cocci, initiating growth by incubation at 37 C. for 24-48 hours,

TABLE 1
THE GROUPS OF THE STAPHYLOCOCCI BASED ON FERMENTATION REACTIONS

Group	Dextrose	Glycerol	Maltose	Lactose	Mannite
I.....	—	—	—	—	—
II.....	+	+	+	—	—
III.....	+	+	+	+	—
IV.....	+	+	+	—	+
V.....	+	+	+	+	+

TABLE 2
THE PROPORTION OF STAPHYLOCOCCI FROM EACH SOURCE FALLING IN EACH FERMENTATION GROUP

Group	Normal Persons, Percentage	Persons with Colds, Percentage
I.....	8.6	5.0
II.....	0.0	21.0
III.....	15.0	42.0
IV.....	12.8	13.8
V.....	61.0	17.0
Miscellaneous.....	2.6	1.2

and leaving at room temperature for 18-21 days. The strains isolated from colds were active in about the same proportion (59% positive) as those isolated from normal throats (54% positive).

Cultivation of the staphylococci in litmus milk for 15-18 days at 37 C. resulted in 3 principal reactions—acid, acid with coagulation, or no change. A few strains produced an alkaline reaction, but the number of strains having this reaction was very small. The reaction in this medium did not differentiate in any way the cocci from the colds.

The sugars employed in the fermentation tests were those recommended by Gordon,¹³ with the addition of dextrose. Lactose, maltose, mannite, glycerol, and dextrose were used in 1% concentration in veal

¹³ 33rd Ann. Rept., Local Gov. Boards for 1903-04, p. 388.

infusion agar, with Andrade's indicator. Inoculations were made on the slant and into the butt. Incubation was at 37 C. for 2-4 days. Reactions of acid without gas or no acid were found complete within that time. On the basis of their reactions, the staphylococci fell into several classes, but chiefly into 5 large groups. These principal groups are shown in table 1.

The difference in distribution of the staphylococci from the two conditions is shown in table 2.

TABLE 3
SUMMARY OF REACTIONS OF THE STAPHYLOCOCCI FROM THE TWO CONDITIONS

Test	Normal				Colds			
	Positive		Negative		Positive		Negative	
	Num-ber	Per-centage	Num-ber	Per-centage	Num-ber	Per-centage	Num-ber	Per-centage
Incidence.....	65	52	60	48	78	80	20	20
Chromogenesis:								
Staph. aureus.....	28	40	34	18		
Staph. albus.....	40	57	146	76		
Staph. citreus.....	1	5	..		
Colorless.....	1	7	..		
Hemolysis (72 hours)	51	73	19	27	113	59	79	41
Liquefaction of gelatin.....	38	54	32	46	112	59	80	41
Litmus Milk:								
Acid (only).....	27	38	59	30		
Acid and coagulation.....	31	45	73	38		
No change.....	11	15	48	25		
Fermentations:								
Dextrose.....	64	91	6	9	182	95	10	5
Glycerol.....	64	91	6	9	172	90	20	10
Maltose.....	63	90	7	10	173	90	19	10
Lactose.....	55	78	15	22	112	58	80	42
Mannite.....	53	76	17	24	62	33	130	67
Indol *.....	70	100	154	100
Nitrate reduction *...	54	77	16	23	91	71	37	29

* These tests were conducted some time after the completion of the other tests, and some of the strains of the group of persons with colds had been lost.

It is evident that the strains from normal persons fermented lactose and mannite more commonly than those from the group of persons suffering with colds.

Indol tests were made (paradimethylamidobenzaldehyde method) after incubation of the staphylococci in peptone broth for 3-5 days at 37 C. Indol was not found in the cultures of any of the staphylococci. This finding agrees with the recently published works of Bayne-Jones and Zininger,¹⁴ Julianelle,¹⁵ and Winslow, Rothberg and Parsons.¹⁶

¹⁴ Bull. Johns Hopkins Hosp., 1921, 32, p. 299.

¹⁵ Jour. Infect. Dis., 1922, 31, p. 256.

¹⁶ Jour. Bacteriol., 1920, 5, p. 145.

Reduction of nitrate to nitrite was determined by the technic prescribed in the 1917 edition of the Standard Methods of Water Analysis (A. P. H. A.). About 75% of the strains were positive for this test, but no significant difference in the groups was noted.

The various reactions of the staphylococci are shown in table 3. It is apparent that there is a difference between the 2 groups in only 3 respects, namely, incidence, proportion of the chromogenic groups, and fermentation of mannite. The greater incidence of this organism in colds may be of some significance, although it does not necessarily imply any primary relation to colds.

THE CLASSIFICATION OF STAPHYLOCOCCI RECOVERED FROM NORMAL PERSONS AND FROM PERSONS WITH COMMON COLDS

There have been many attempts to find a rational and dependable basis of classification for the staphylococci, but difficulties have arisen because of the many variations in this group without sharp lines of division. The first important groupings of the cocci were proposed by Migula¹⁷ and Chester,¹⁷ who considered general morphological and cultural characteristics. The Winslows¹⁷ rearranged all the cocci and added the consideration of pathogenicity and principally chromogenesis. Their grouping was supported by the work of Kligler¹⁸ on a limited number of strains.

The inconstancy and difficulty of determination of chromogenesis led to a search for classification along cultural lines. This resulted in the schemes of Gordon,¹³ Cummins and Cumming,¹⁹ Marbais,²⁰ Dudgeon,²¹ and Winslow, Rothberg, and Parsons.¹⁶ The last system groups all staphylococci as one genus, subdivided according to chromogenesis as well as cultural reactions.

Kolle and Otto²² affirm the importance of the separation of the staphylococci on the basis of pathogenicity and divided pathogenic from nonpathogenic strains by means of agglutination tests. Geisse²³ considered this a reliable means of determining a pathogenic or a nonpathogenic strain. This serologic test was used by Walker and

¹⁷ The Systematic Relationships of the Coccaceae, 1908.

¹⁸ Jour. Infect. Dis., 1913, 12, p. 432.

¹⁹ Jour. Royal Army Med. Corps., 1913, 20, p. 499.

²⁰ Compt. rend. de la Soc. de Biol., 1919, 82, p. 220.

²¹ Jour. Path. & Bacteriol., 1908, 12, p. 242.

²² Ztschr. f. Hyg. u. Infektionkr., 1902, 41, p. 369.

²³ Ibid., 1913, 76, p. 283.

Adkinson,²⁴ who concluded that it was the best and quickest method of differentiating the kinds of staphylococci.

The tests used in this attempt to effect a classification of staphylococci from the upper respiratory tract were partly those used in the first part of this work in connection with the relation of this organism to colds.

In addition to these tests, agglutination and precipitin reactions were employed to determine serologic relations. Rabbits were immunized against strains representing chromogenic and at the same time cultural groups (table 1). Homologous and cross serologic tests were repeatedly made with the immune serums and the various antigens. Serums consistently agglutinating the homologous strain in dilutions as high as 1:1,600 were obtained. But strains similar to the antigens either culturally or chromogenically were not clumped regularly by the immune serums, although positive reactions often occurred. Suspensions of *Staph. aureus* cultures were usually, but not always, agglutinated by antiserums against strains of different as well as the same chromogenic group.

Precipitin tests were conducted with antigens prepared by the growth of the staphylococci in broth for 30 days, and clearing by rapid centrifugation. Other methods for the preparation of the antigen were unsatisfactory. Antigens prepared by long incubation were precipitated by homologous serums only in the low dilution of 1:10. The agglutination and precipitin tests proved to be unsatisfactory as means of classification of this organism.

It seemed that after all, chromogenesis was the most practical and rational basis of correlation. It was found that when the cocci from the 2 sources were grouped according to chromogenesis, 62 or 24% of the 262 strains produced golden pigment, 186 or 71% produced a white pigment, 6 strains were lemon yellow, and 8 were colorless. The divisions were not sharp, and it was evident that the staphylococci represent a group varying in the ability to produce pigment from a rich golden color to none.

When the reactions on various mediums were compared with pigment production, it was found that the group rich in color was more actively metabolic than the other groups. The *Staph. aureus* group hemolyzed blood in blood-agar plates in a larger number of cases and more quickly than *Staph. albus*. It was a little more active in liquefy-

²⁴ Jour Med. Res., 1917, 35, p. 373.

ing gelatin and acidifying milk, but slightly less active in coagulating milk. A larger number of strains of *Staph. aureus* than of *Staph. albus* reduced nitrate to nitrite.

The sugar reactions did not offer a differentiation between the two principal chromogenic groups, except in the speed with which glycerol was attacked and the ability to ferment mannite. Even these reactions were not sharp; 92% of the strains of *Staph. aureus* fermented glycerol only after 48 hours, while 13% of the strains of *Staph. albus* were

TABLE 4
THE DISTRIBUTION OF THE CHROMOGENIC GROUPS AMONG THE PRINCIPAL FERMENTATION GROUPS

Group	Fermentation Group					Chromogenic Group	No. of Strains	Percentage of All Strains
	Dex-trose	Gly-cerol	Mal-tose	Lac-tose	Man-nite			
I	—	—	—	—	—	<i>Staph. citreus</i> and colorless..	11	6
						<i>Staph. albus</i>	5	
							16	
II	+	+	+	—	—	Colorless.....	1	15
						<i>Staph. albus</i>	33	
						<i>Staph. aureus</i>	5	
							39	
III	+	+	+	+	—	Colorless.....	1	33
						<i>Staph. albus</i>	82	
						<i>Staph. aureus</i>	4	
							87	
IV	+	+	+	—	+	<i>Staph. albus</i>	27	13
						<i>Staph. aureus</i>	7	
							34	
V	+	+	+	+	+	<i>Staph. albus</i>	30	28
						<i>Staph. aureus</i>	44	
							74	

similarly slow in fermenting this alcohol. *Staph. aureus* produced acid in glycerol in 24 hours in 8% of the strains, and *Staph. albus* in 78%. A larger number of strains of *Staph. aureus* (84% positive) fermented mannite than *Staph. albus* (34% positive). With the exception of these differences, there were no sharp lines between the chromogenic groups. The few strains of *Staph. citreus* were found to attack no sugars, and the colorless strains were much like *Staph. citreus*, although a few produced acid from the simpler sugars. There were also a few strains (5) of *Staph. albus* that did not ferment any of the sugars resembling *Staph. citreus* in this respect.

Lactose and mannite were found to be the differentiating carbohydrates since if the staphylococci fermented any at all, they fermented dextrose, maltose, and glycerol. With the exception of 12 strains that fell into minor groups, the majority of the strains were found to be in the 5 large groups, according to the scheme of table 1. The distribution of the chromogenic groups among the principal fermentation

TABLE 5
THE REACTIONS OF THE TWO PRINCIPAL CHROMOGENIC GROUPS IN THE VARIOUS TESTS CONDUCTED

Test	Chromogenic Groups			
	Staph. aureus		Staph. albus	
	No. of Strains	Per-centage	No. of Strains	Per-centage
Chromogenesis.....	62	24	186	71
Hemolysis (72 hours).....	Pos. 50	81	107	58
	Neg. 12	19	79	42
Gelatin liquefaction.....	Pos. 42	68	103	55
	Neg. 20	32	83	45
Litmus Milk:				
Acid only.....	Pos. 53	53	53	28
Acid and coagulation.....	Pos. 22	35	82	44
No change.....	7	11	51	27
Indol *.....	Pos. —	—	—	—
	Neg. 59	100	151	100
Nitrate reduction *.....	Pos. 50	94	94	65
	Neg. 3	6	50	35
Fermentation of Carbohydrates:				
Dextrose.....	Pos. 62	100	181	97
	Neg. —	—	5	3
Glycerol, 24 hours.....	Pos. 5	8	146	78
48 hours.....	Pos. 57	92	24	13
total.....	Pos. 62	100	170	91
	Neg. —	—	16	9
Maltose.....	Pos. 62	100	171	92
	Neg. —	—	15	8
Lactose.....	Pos. 49	79	116	62
	Neg. 13	21	70	38
Mannite.....	Pos. 52	84	63	34
	Neg. 10	16	123	66

* These tests were conducted some time after the completion of the other tests, and some of the strains of the group of persons with colds had been lost.

groups is shown in table 4. The reactions of the chromogenic groups in all the tests conducted are summarized in table 5.

SUMMARY

Staphylococci were grown from the nasopharynx and throat in a higher percentage of persons with common colds than of normal persons. A higher proportion of Staph. aureus was found in normal

persons than in persons suffering with colds. Staphylococci are probably opportunists that multiply more freely or invade the tissue when the resistance of the host is lowered by some other organism or virus.

There was no marked distinction in the biochemical reactions between the staphylococci from persons with colds and those from normal persons, except that mannite was more commonly fermented by strains from the normal source. Staphylococci showed remarkably similar reactions in the various tests employed. There seemed to be no group of staphylococci in the upper respiratory tract peculiar to the condition of colds.

The attempt to classify the staphylococci recovered from the upper air passages of man disclosed that there was great variation in biochemical and serologic activity, with constantly appearing gradations. Chromogenesis, although often an unsatisfactory basis, was decided as, on the whole, the most practical. On this basis there were found the golden, the white, the lemon yellow and the colorless groups. *Staph. aureus* was the most active in producing biochemical changes. *Staph. albus* next less active, the colorless group next, and *Staph. citreus* the least active.

No strains of the staphylococcus formed indol after culture in peptone broth for 3-5 days. Serologic tests were unsuccessful as means of correlating members of chromogenic or fermentation groups. Highly specific antisera were produced by the immunization of rabbits, that agglutinated the homologous strains in dilutions from 1:800 to 1:1,600 but failed to agglutinate regularly any heterologous strains.

The presence of chromogenic groups, the indefinite differential lines, and the existence of intermediate forms between either the chromogenic or fermentation groups, and the greater biochemical activity of *Staph. aureus* are points that favor the view of Winslow, Rothberg, and Parsons¹⁶ that the staphylococci belong to one generic group, that they can logically be subdivided on the basis of chromogenesis, and that *Staph. aureus* represents the main type from which *Staph. albus* and *Staph. citreus* have varied in chromogenic and cultural powers.

STUDY OF BACTERIAL PRODUCTS BY MEANS OF EXCISED MAMMALIAN HEART

I. ENDOTHELIOTOXIN OF *S. CHOLERAE*

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The excised mammalian heart is a delicate reacting index of toxic action. It was thought that tests with this organ might throw light on the physiologic action of certain bacterial products, giving little or no recognizable reactions in intact animals.

The technic used in making these tests is the simplified technic proposed by Gunn¹ and described in a previous paper.² The preferable perfusion fluid for the excised rabbit heart is well aerated Locke's solution containing 1% to 2% carefully filtered defibrinated blood from the same animal. A normal rabbit heart perfused with this mixture beats regularly and strongly for about 3 hours.

To prepare *S. cholerae* products for these tests, the microorganisms were grown in a specially prepared broth, containing 1% peptone, 0.25% beef extract, and 0.5% NaCl, and having a reaction identical with that of Locke's solution (0.015% NaHCO₃). The cultures were incubated in sealed flasks to prevent evaporation, and then passed through a Berkefeld filter. To each unit volume of the filtrate there was added one volume of a correction fluid, containing 1.3% NaCl, 0.015% NaHCO₃, 0.084% KCl, 0.048% CaCl₂ and 0.2% dextrose. The resulting mixture was Locke's solution, plus 0.5% peptone and 0.125% beef extract, with such modifications and additions as were produced as the result of bacterial growth.

This culture medium in itself is slightly toxic for the excised rabbit heart. Control hearts, however, perfused with 10% uninoculated culture medium beat regularly and strongly for over an hour, at which time our tests were usually discontinued. There is no demonstrable myocardial edema.

The toxic action of cholera filtrates will be presented under two headings: (1) action on the cardiac conducting and contractile tissues, (2) action on the cardiac blood vessels.

ACTION ON THE CONDUCTING AND CONTRACTILE TISSUES

Two- to seven-day cholera filtrates, tested in from 5% to 10% dilution in Locke's solution, are almost nontoxic for the cardiac con-

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¹ Jour. Physiol., 1913, 46, p. 506.

² Jour. Immunol. (in press).

ducting and contractile tissues. Minor changes in rate and strength of the myocardial contractions are noted, but the hearts beat regularly and strongly for 90 minutes, at which time our tests were discontinued. A typical slow-drum record is shown in fig. 1. We believe the fall in the base line in this record is not due to toxic action on the contractile tissues (see below).

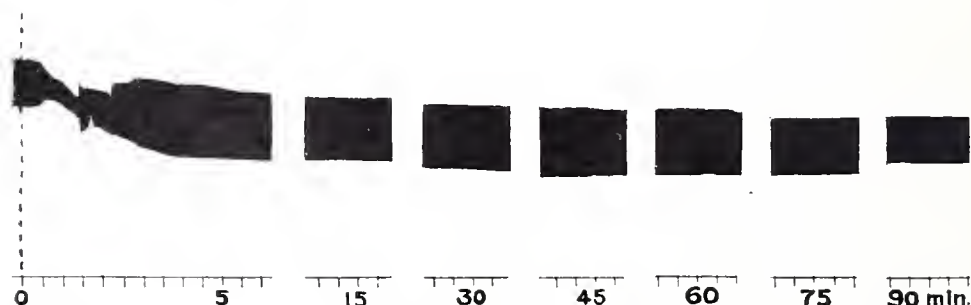


Fig. 1.—Action of cholera filtrate on the excised rabbit heart. Perfusion fluid: 6%, 4-day cholera filtrate plus 2% defibrinated rabbit blood. Vertical dotted line shows time of changing the perfusion clamps. The fall in the base line is believed to be due solely to myocardial edema.

ACTION ON THE BLOOD VESSELS

Striking reactions, however, are produced on the capillary endothelium. Within 10 minutes after beginning the perfusion, the myocardium becomes markedly edematous. We believe the fall in the base line above noted is due to this edema. Within 30 minutes, the hearts become markedly hemorrhagic, often taking on the appearance of coagulated blood. Histologic examinations of these hearts show the muscular elements widely separated by edematous fluid, with few extravasated red blood corpuscles in the tissue spaces. Immediately beneath the pericardium and endocardium, however, numerous closely packed extravasated red blood corpuscles are seen. The extravasated corpuscles apparently migrate to the myocardial surfaces as a result of the rhythmic changes in tissue pressure.

We believe this action of cholera filtrate is in line with clinical symptoms and necropsy findings. A more detailed study of this endotheliotoxin will be reported later.

SUMMARY

Filtrates from broth cultures of *S. cholerae* are almost nontoxic for the conducting and contractile tissues of the excised rabbit heart.

The filtrates, however, are markedly toxic for the capillary endothelium, producing striking myocardial edema and diapedesis.

STUDY OF BACTERIAL PRODUCTS BY MEANS OF EXCISED MAMMALIAN HEART

II. SUBHEMAGGLUTININ, ENDOTHELIOTOXIN AND MYOTOXIN OF STREPTOCOCCUS HEMOLYTICUS

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To prepare streptococcus products for this study the micro-organisms were grown in 10% defibrinated rabbit blood in Locke's solution, by the method of Clark and Felton.¹ The culture flasks were sealed to prevent evaporation during incubation. After incubation, the cultures were centrifuged at our highest speed and the supernatant fluid decanted. Since streptococci grow in this medium mainly in masses adherent to the agglutinated red blood corpuscles, the fluid thus obtained was practically free from micro-organisms.

This dilute blood in itself is slightly toxic for the excised rabbit heart. The toxicity varies with the length of incubation and the amount of hemoglobin set free in the supernatant fluid. With the most toxic uninoculated culture medium thus far obtained, however, rabbit hearts beat regularly and strongly for over an hour. Our streptococcus tests were usually limited to 45 minutes, during which time the uninoculated culture medium gives negligible reactions.

The toxic action of the streptococcus products will be presented under four headings: (1) action on the red blood corpuscles, (2) action on the blood vessels, (3) action on the myocardial conducting tissues, and (4) action on the myocardial contractile tissues.

ACTION ON THE RED BLOOD CORPUSCLES

By our routine technic, the hearts are perfused with a 25% to 50% dilutions of streptococcus centrifugate in Locke's solution plus 1% to 2% defibrinated rabbit blood. In our earlier tests, we uniformly observed a rapid decrease in the rate of perfusion flow with this mixture, the perfusion often practically ceasing in 5 minutes. Histologic examinations of the earlier hearts show practically all of the cardiac arterioles plugged with agglutinated red blood corpuscles.

Received for publication, Jan. 24, 1923.

¹ Jour. Am. Med. Assn., 1918, 71, p. 1048.

Hemagglutination in vitro was observed only once in these dilute blood mixtures. If, however, the blood mixtures were centrifuged, a distinctly increased adhesiveness of the red blood corpuscles was always demonstrable. We therefore believe that the perfusion method is a more delicate test of hemagglutination than the routine test-tube reaction, and that hemagglutination plays a more important rôle in streptococcus pathogenicity than is currently assumed.

The process of intravascular hemagglutination is undoubtedly favored by capillary vasoconstriction (see below) and presumably favored by alterations in the capillary endothelium. We suggest the

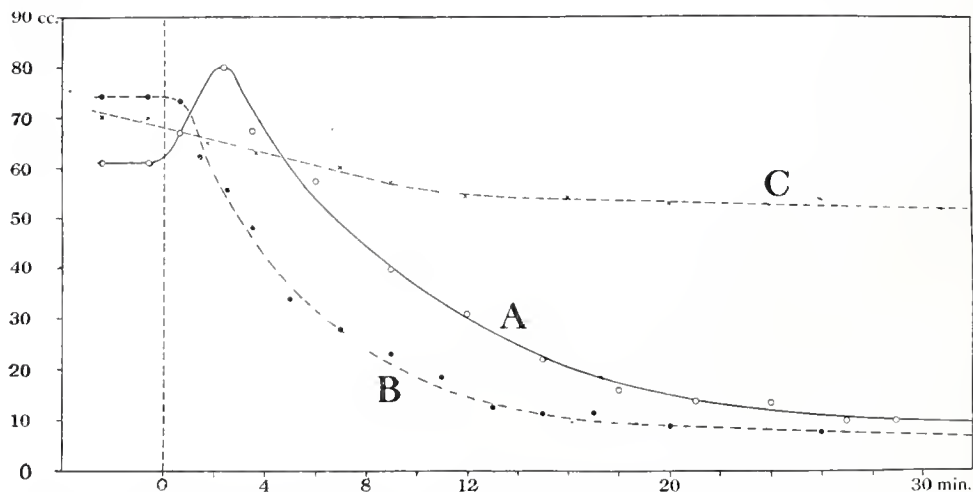


Fig. 1.—Changes in perfusion rate. *A*, *B* indicate perfusions with 25%, 48- to 96-hour streptococcus centrifugate, plus 1.5% defibrinated blood. There was no intravascular hemagglutination. *C* indicates control perfusion with 25% uninoculated culture medium. The vertical dotted line shows time of changing the perfusion clamps. Data show perfusion rates per minute; perfusion pressure, 50 mm. Hg.

term subhemagglutination to designate the increased corpuscle adhesiveness essential to this phenomenon.

The streptococcus used in the earlier tests was a stock culture of low pathogenicity. To increase its virulence, the strain was repeatedly passed through rabbits and grown in ground rabbit meat medium and in the presence of defibrinated rabbit blood. After three animal passages, the strain ceased to give intravascular hemagglutination. This change is in line with the observations of Howell,² who reports a similar loss of hemagglutination in her streptococcus cultures. Unless otherwise stated, the later nonhemagglutinating streptococcus strain was used in all tests hereinafter reported.

² Jour. Infect. Dis., 1920, 27, p. 565.

ACTION ON THE BLOOD VESSELS

In control perfusions with 25% to 50% uninoculated culture medium plus 1% to 2% rabbit blood, the rate of perfusion flow remains fairly constant. The perfusion rate at the end of half an hour is usually at least 75% of the initial rate (*C*, fig. 1). Two- to four-day streptococcus cultures, however, similarly tested, invariably produce a rapid decrease in perfusion rate, the flow usually being reduced to about 15% of the initial rate by the end of 12 minutes (*B*, fig. 1). Histologic examinations of these hearts show no plugging of the arterioles with agglutinated red blood corpuscles, and negligible edema. There is decreased myocardial tone (see below). The marked reduction in perfusion rate therefore is evidently due to active vasoconstriction.

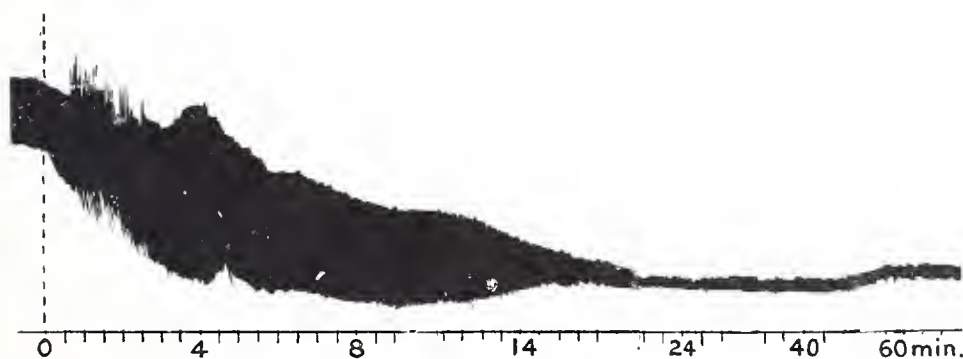


Fig. 2.—Changes in myocardial tone. Perfusion with 25%, 48-hour streptococcus centrifugate, plus 1.5% defibrinated blood; no intravascular hemagglutination.

In our histologic preparations the arterioles are widely dilated and the capillaries collapsed. We believe, therefore, that the reduction in perfusion rate is probably due to capillary reactions. If a similar capillary vasoconstriction takes place in local tissues during certain stages of streptococcus infection, it must be an important factor in the mechanism of streptococcus pathogenicity.

Capillary permeability is only slightly increased by our streptococcus products, as shown by the absence of marked edema. Diapedesis was not observed.³

ACTION ON THE MYOCARDIAL CONDUCTING TISSUES

Two- to four-day streptococcus cultures invariably produce a temporary, partial or complete auricular-ventricular dissociation (heart-

³ Compare cholera filtrates, Jour. Infect. Dis., 1923, 32, p. 407.

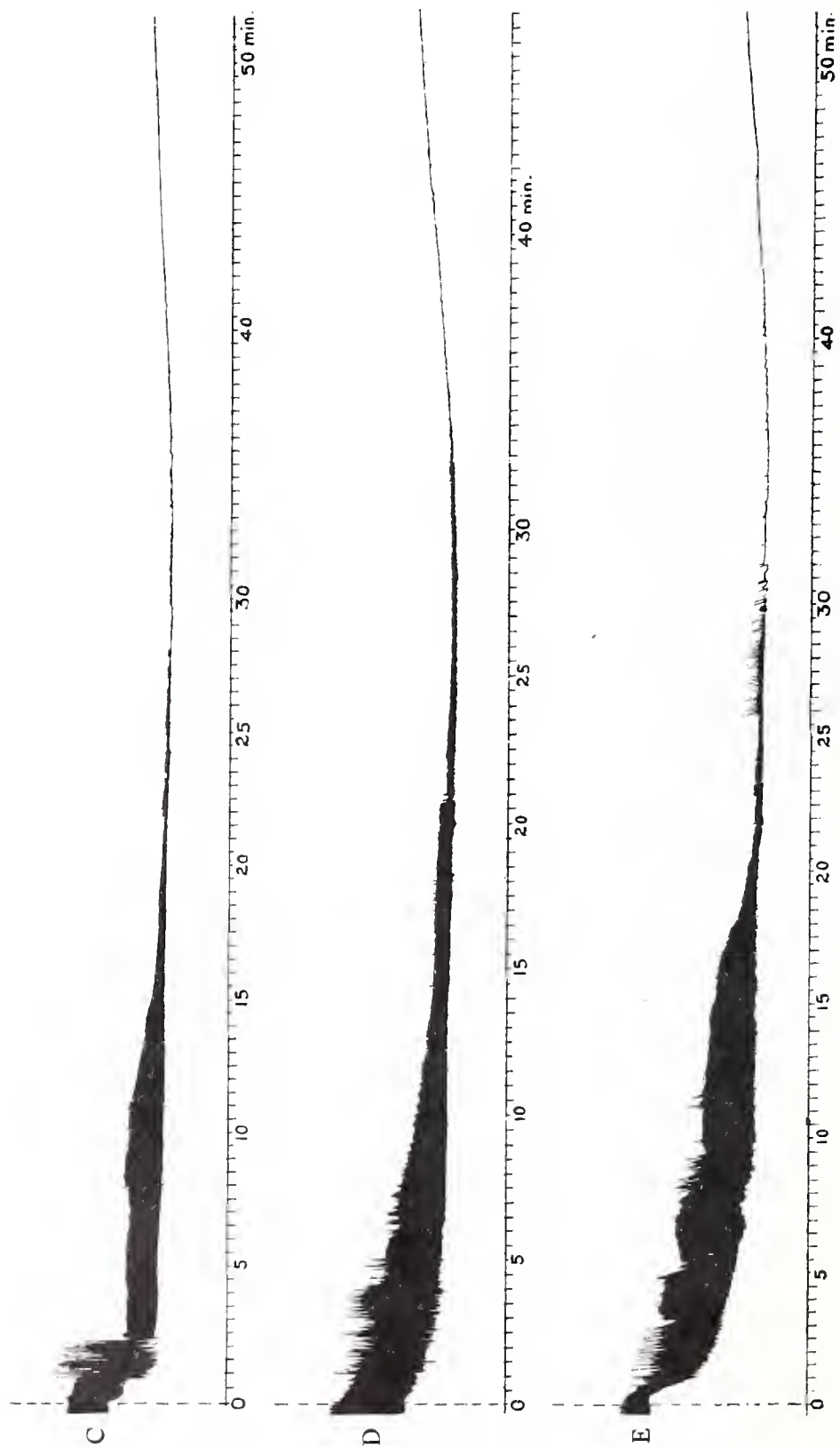


Fig. 3.—Typical myocardial reactions. *C* indicates 50%, 48-hour streptococcus centrifugate, plus 1.5% defibrinated blood. Complete heart block begins in 45 seconds and lasts 90 seconds; no intravascular hemagglutination. *D* indicates 50%, 7-day streptococcus centrifugate, plus 1.5% defibrinated blood. Complete heart block begins in 30 seconds and lasts 5 minutes; no intravascular hemagglutination. *E* indicates 25%, 48-hour streptococcus centrifugate, plus 2% defibrinated blood. Partial heart block, with 1:2 auriculoventricular rhythm begins in 30 seconds, changes to a 1:3 auriculoventricular rhythm at the end of 4 minutes. Normal auriculoventricular rhythm resumed at the end of 6 minutes; no intravascular hemagglutination.

block). This reaction usually begins about 30 seconds after commencing the streptococcus perfusion, and usually lasts from 3 to 5 minutes (*D*, fig. 3). At the end of this period the normal auricular-ventricular association is suddenly resumed and continues until the terminal stages of the test. Since in certain of our tests (*E*, fig. 3) there was established a definite 1:2 and 1:3 auricular-ventricular rhythm during this period, we believe the reaction is due to depression of the myocardial conducting system.

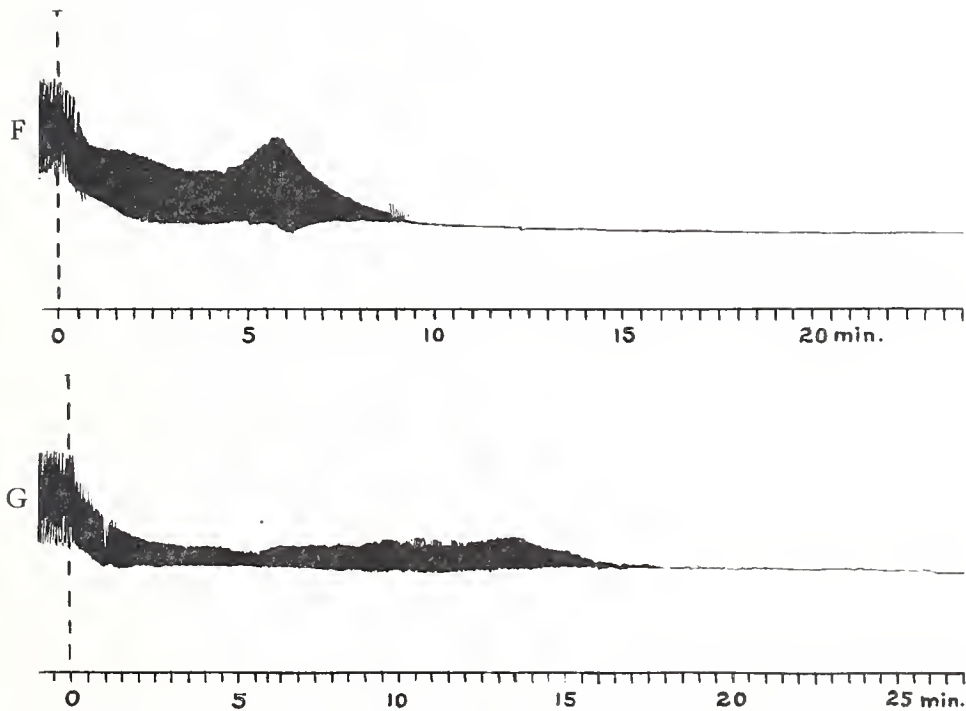


Fig. 4.—Maximum toxicity. *F* indicates 50%, 96-hour streptococcus centrifugate, plus 2% defibrinated blood. *G* indicates 50%, 48-hour streptococcus centrifugate, plus 2% defibrinated blood. Intravascular hemagglutination in both cases.

ACTION ON THE MYOCARDIAL CONTRACTILE TISSUES

Auricles.—The auricular rhythm is usually regular throughout the earlier stages of the test. A marked auricular arrhythmia, however, is occasionally observed.

Ventricles.—Two- to four-day streptococcus cultures invariably produce: (*a*) Marked loss of ventricular tone, (*b*) decreased strength of ventricular contractions, together with (*c*) changes in rate not considered in this paper.

The loss of ventricular tone usually begins immediately on commencing the streptococcus perfusion (fig. 2), and usually reaches a

maximum in about 5 minutes. The ventricular relaxation is greatest with relatively nontoxic streptococcus products. At the height of the reaction, the heart is enormously dilated, the right ventricle hanging down as a flabby bag distended with escaped perfusion fluid. During the final stages of the test, a partial restoration of tone is usually observed (*D*, fig. 3). Histologic examinations of the dilated hearts show only slight edema.

The decreased strength of the ventricular contractions is usually noticeable in from 5 to 10 minutes after beginning the streptococcus perfusion (*D*, fig. 3). It is occasionally observed as early as in 3 minutes (*C*, fig. 3). It usually leads to complete ventricular standstill in from 25 to 30 minutes (fig. 3). With our earlier hemagglutinating streptococcus strain, intravascular hemagglutination with complete ventricular immobilization was produced in from 10 to 20 minutes (fig. 4).

CONCLUSION

We believe the actions of streptococcus centrifugates noted in the foregoing are in line with clinical observations and necropsy findings. A more detailed study of these products will be reported later.

SUMMARY

Streptococcus hemolyticus grown in 10% defibrinated rabbit blood in Locke's solution gives products producing the following toxic reactions on the excised rabbit heart: (*a*) intravascular hemagglutination; (*b*) marked capillary vasoconstriction; (*c*) slightly increased capillary permeability; (*d*) auriculoventricular dissociation (heart-block); (*e*) marked loss of ventricular tone, and (*f*) reduction in the strength of the ventricular contractions, leading to cessation of recordable movements in from 25 to 30 minutes.

We believe these reactions are in line with clinical observations and necropsy findings.

A STUDY OF RABBIT SPIROCHETOSIS *

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The fact that a number of investigators in different countries have noted the occurrence in rabbits of a spontaneous venereal spirochetosis resembling syphilis, in the lesions of which a spirochete said to be "indistinguishable from *Treponema pallidum*" has been demonstrated, is of great importance for two reasons: First, it adds a new spirochete and a new spirochetal disease to our list; second, the existence of a spontaneous syphilis-like disease in rabbits, due to an organism so closely resembling *Spirochaeta pallida*, must throw into question the whole mass of experimental work that has been carried out with reference to the transmission of human syphilis to the rabbit. Has the organism of the primary rabbit infection been mistakenly regarded at any time as *Spirochaeta pallida*?

The literature on spontaneous rabbit spirochetosis is not yet extensive, and the observers who have studied it are relatively few, although widely scattered. It is necessary that the frequency of this spontaneous infection of rabbits be known with certainty, and that the essential characteristics of the organism and the pathologic lesions produced by it be more fully determined in order to dispel the present uncertainty as to the possibility of mistaken identifications of this rabbit disease with true syphilis.

Reviewing briefly the literature, we find that our knowledge of primary venereal spirochetosis in rabbits begins with the observations made by Ross ¹ in 1912, who found parasites similar to those of syphilis in the sores and blood of rabbits exhibiting chancres, buboes, ulcers of the genitalia, mouth and anus. The organisms were numerous in the sores, and he believed they showed phases indistinguishable from those of the human parasite.

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¹ Brit. Med. Jour., 1912, 2, p. 1653.

In 1913, Bayon² described a new species of *Treponema* found in genital sores of rabbits. In the serum expressed from a papulous sore of the penis in a rabbit, he found spirochetes by dark-field examination. From this spontaneous lesion he successfully inoculated another rabbit by scarification with a needle contaminated by serum from the original sore. The inoculated lesion developed in twenty-five days. He described the new organism as one and a half times as long as the diameter of a rabbit erythrocyte, with close curves, numbering 5 to 8; in transverse section the organism was slightly oval; the ends tapered off without terminal flagellae. Transversely dividing forms were seen. The movements were slow and rotatory without lashing. He called the attention of workers on experimental rabbit syphilis to the existence of this new species of *Treponema*.

Arzt and Kerl³ made an extensive study of several broods of rabbits, 853 in all. Excluding the young ones, they found in a total of 267 adult rabbits that 72 (26.9%) showed flat, scaly, papular or ulcerative lesions of the anogenital region containing numerous spirochetes that they could not distinguish morphologically from *Spirochaeta pallida*. Few broods were entirely free from the infection. Swelling of the inguinal glands was noted in four cases; dark-field examination showed a few spirochetes in one case. In one case, a lesion rich in spirochetes was found on the lip. Uninfected animals were successfully inoculated from the spontaneous lesions, developing genital lesions with spirochetes present in 27 days. Intratesticular inoculations were not successful. These authors state that the appearance of lesions on the genitals of rabbits is known to breeders who use compound solution of cresol as a therapeutic measure.

No further observations on the occurrence of spontaneous spirochetal infections of the rabbit appeared until after the war. In 1920, Arzt⁴ again took up the study of this disease. He found spirochetal lesions in 3 out of 6 males, and in 3 out of 7 females examined, while the examination of 16 young ones was negative. These examinations were made in Innsbruck where no experimental researches on rabbit syphilis had ever been made. The lesions in the male were in part on the penis and in part scrotal. The prepuce was edematous and swollen, with partial phimosis; at the root of the penis there were

² Ibid., 1913, 2, p. 1159.

³ Wien. klin. Wchnschr., 1914, 27, p. 1053; Dermat. Wchnschr., 1920, 71, p. 1047.

⁴ Dermat. Ztschr., 1920, 29, p. 65.

areas denuded of epithelium, covered with scabs, and bleeding easily. The scrotal lesions were small circular excoriations or efflorescences. In the females the whole vulva appeared bright red, partly excoriated, the clitoris in some cases showing scales and bleeding freely on irritation. The inguinal glands were not enlarged, and there were no symptoms or lesions outside of the genitalia. Spirochetes were easily demonstrated in the dark-field, by Fontana's silver impregnation method and by Giemsa's stain. These were regarded as morphologically indistinguishable from *pallida*. They were described as fine or delicate threads with corkscrew turns, with whip or terminal flagella, varying in size, 7 to 15 to 20 turns, the turns fine and sharply placed. Variations in form were circles, arches, V or Y forms, no turns in the middle; the V and Y forms rotated lengthwise. Arzt also successfully inoculated with *pallida* the testicle of a rabbit infected with spontaneous spirochetosis. Since immunity is inconstant or lacking in syphilitic rabbits to double infection or superinfection, differentiation of the two infections cannot be secured in this way, nor can the Wassermann reaction be applied for such purpose.

In the same year, Jacobsthal⁵ reported observations on a syphilis-like spontaneous rabbit disease, a typical case of which he had seen during the preceding year in a female rabbit with erosions of the vulva and shank. The glands were not enlarged. The secretion from the lesions showed numerous spirochetes at first glance not distinguishable from *pallida*, a trifle thicker and without the undulating motion of *pallida*. They stained like *pallida* in smears. Their length was similar; their turns varied from 8 to 14, the average number being 10 to 12. By means of light scarification on the vulva with infective material, the infection was successfully inoculated in 3 out of 4 females, the lesion developing in 3½ to 4 weeks. In one of these, a chancre-like lesion was produced. Inoculation of testicles was unsuccessful, all of the animals dying of peritonitis; corneal inoculation was also unsuccessful. The infection could not be transmitted to mice and guinea-pigs. In sections of the primary lesion, the Levaditi method gave poor staining results; only a few spirochetes were found around some of the vessels. The histologic picture was that of a superficial ulceration with deep round cell infiltration. Jacobsthal believed the infection could be distinguished from true syphilis by the incubation period and the differ-

⁵ Dermat. Wehnschr., 1920, 71, p. 569.

ence in the pathologic picture. He proposed that the disease be called paralues cuniculi and the infective organism *Spirochaeta paralues cuniculi*.

In another paper, Arzt and Kerl again take up the question of the differentiation of rabbit syphilis and the spontaneous venereal spirochetosis, referring to their inoculation experiments in 1914. They conclude that neither the inoculation period nor the histologic changes of the lesions warrants a differential diagnosis from experimental syphilis in the rabbit.

In the same year, Schereschewsky⁶ described the chief features of the primary venereal rabbit spirochetosis. The genital lesions were papular or ulcerative, and contained numerous spirochetes showing all of the characteristics of pallida. The incubation period was like that of syphilis, 14 to 30 days. The earliest stage of the lesion was often a scarcely perceptible macule containing spirochetes; this developed through stages of infiltration, papule formation, ulceration and scab formation, the process tending to remain local. In one case only did keratitis develop, 2 months after the onset. In 3 cases, a scaly papule appeared on the lip. The infection could be transferred by coitus. Spirochetes were found in the seminal fluid. Successful inoculation could be made by light scarification. Impregnation of a sick female by a sick male gave living young. In a few rabbits the liver and spleen were enlarged, but no spirochetes were present. In a few cases, crusted lesions persisted for more than a year. Fading lesions could be made to flare up by mechanical irritation or coitus. Apes showed no lesion 25 days after inoculation with the rabbit spirochetes. The rabbit organism is like pallida as far as the winding, number of turns and motion are concerned. It may be killed by quinine ointment.

In 1921, Klarenbeek⁷ studied this disease in 5 rabbits found in Holland, showing papular, red and scaly, easily bleeding genital lesions in which numerous spirochetes indistinguishable from pallida were demonstrated by the dark-field and Fontana's silver method. Spirochetal material inoculated into genital scarifications gave positive results in from 11 days to 3 to 4 weeks. Scarification of the dorsal skin gave positive results in 4 out of 6 cases in from 1 to 2 months. Subcutaneous inoculations in the upper eyelid caused ulcers after 5 to 8 weeks; the ulcers were small, round, covered with white scales which later became

⁶ Berl. klin. Wchnschr., 1920, 57, p. 1142.

⁷ Ann. de l'Inst. Pasteur, 1921, 35, p. 326.

dark. Intra-ocular inoculation produced ulcers on the upper eyelid and inflammation of the cornea. Intratesticular inoculation was positive in 3 cases out of 8, giving rise to nodules containing spirochetes. Intravenous injections gave no results; death occurred from other infections. General infection occurred 6 weeks after intra-ocular inoculation in lesions of the skin near the ear, eyes and nose; the rabbit remained in good health. Klarenbeck regards the identity of the appearance of this spirochete with that of the pallida as rendering all assertions concerning the occurrence of pallida in rabbits untrustworthy.

In 1921, Noguchi⁸ published a note on venereal spirochetosis in rabbits, suggesting the name *Treponema cuniculi* for the organism, and giving a new staining method for *Treponema pallidum*. In 1922,⁹ he published the first observation of the occurrence of spontaneous rabbit spirochetosis in American rabbits. Of 50 rabbits examined in June, 1921, three females and two males were found to have lesions on the vulva, prepuce and perineum. In November, 1921, six females with similar lesions were found in twenty rabbits obtained from Pennsylvania. The genitoperitoneal lesions were described as papulosquamous, often ulcerating, running a chronic course, and characterized by the presence of a spiral organism closely resembling *Treponema pallidum*. The rabbit spirochetes were described as morphologically resembling pallidum, possibly a trifle thicker and larger than the average pallidum. Long specimens measuring 30μ were common, showing a tendency to form loosely entangled knots. A stellate arrangement of several organisms in a mass was frequently seen. Coarser variant forms were found in one rabbit. The disease was found to be readily transmissible to normal rabbits, the inoculation period varying from 5 to 20 to 88 days. No typical orchitis or keratitis was produced; in one of the original rabbits, scaly, papular lesions developed on the nose, lips, eyelids and paws. Monkeys failed to show any lesions within a period of 4 months after inoculation. In one instance, transmission was accomplished through the mating of an infected female with a normal male. The Wassermann reaction was uniformly negative. Arsphenamin had the same therapeutic effect on the lesions produced by the rabbit spirochete as on the experimental pallidum lesions of the rabbit. Noguchi regards the histologic reactions as similar to, but considerably less cellular than, those occurring in typical primary syphilitic lesions, but with a marked hyperkeratosis and interpapillary infiltration not observed in scrotal

⁸ Jour. Am. Med. Assn., 1921, 77, p. 2052.

⁹ Jour. Exp. Med., 1922, 35, p. 391.

chancre. He regards the organism as belonging to the *Treponema* genus, and suggests that it be called *Treponema cuniculi*.

The latest communication on the subject of primary rabbit spirochetosis is that of Kolle and Ruffer.¹⁰ They attack the problem from the standpoint of the chemotherapeutic differentiation of *Spirochaeta pallida* and *Spirochaeta cuniculi* in rabbits. They state that Levaditi, Marie and Isaïen¹¹ found that *spirochaeta cuniculi* was not infectious for man. Primary *pallida* lesions in rabbits heal and the spirochetes disappear when treated with 4 mg. per kilo bodyweight of silver arsphenamin. No effect is produced on *Spirochaeta cuniculi* with the same dosage, or even 5 or 6 mg. per kilo bodyweight of silver arsphenamin. More than 7 mg., usually 10 mg., will cure the lesion. In rabbits infected with both *pallida* and *cuniculi*, a dosage of 4, 5 or 6 mg. per kilo bodyweight, of silver arsphenamin will cause *pallida* to disappear and the chancre to heal, while the *cuniculi* will still persist in flat erosions.

These contributions constitute the mass of our knowledge relating to this spirochetal disease of rabbits at this time. From this it will be seen that all of the investigators, with the exception of Jacobsthal have emphasized the close similarity of the *cuniculi* organisms and lesions to *pallida* and the rabbit lesions produced by it. The value of all the experimental work on rabbit syphilis is, therefore, challenged; and more complete studies of the spontaneous spirochetosis of rabbits are made imperative at this time in order to give experimental rabbit syphilis a more secure footing. It is evident from the foregoing resumé that no adequate pathologic study has yet been made of the *cuniculi* disease. Further, the organisms themselves have been studied chiefly by the dark-field, which offers less opportunity for morphologic differentiation than do properly prepared and stained cover-glass preparations. The purpose of this paper is to present the result of a more detailed study of the *cuniculi* disease than has yet been made, basing this study on the disease as produced experimentally in rabbits by inoculation from a spontaneous case found in a Western rabbit.

THE MATERIAL

The present report is based on a study of *Spirochaeta cuniculi* and the lesions produced by it in 18 rabbits showing the infection. Only one of these was a spontaneous case, the original infected rabbit found by Dr. A. C. Starry in Denver. All of the other cases, with the exception of rabbit R, were inoculated with the strain derived from this case, either directly from it, or after

¹⁰ Med. Klin., 1922, 18, p. 620.

¹¹ Compt. rev. de la Soc. de biol., 1921, 85, p. 51.

passing through one or more animals. In the case of rabbit R, the disease was transmitted by coitus. Before inoculation the anogenital regions of the animals were carefully examined for the presence of spirochetes, but none were found in any normal animal.

The successful inoculations were made by scarification, with 4 exceptions. In these 4 cases only did inoculation without scarification succeed, and it is noteworthy that in these cases the inoculation period was very much prolonged being 56 days in 2 cases, and 27 and 28 days in the other 2. Intravenous inoculation was not successful.

Rabbit A.—A large gray and white female was discovered by Dr. A. C. Starry in the pens of a rabbit breeder in Denver. In February, after delivery, the rabbit had developed a scaly papular lesion on the vulva which showed spirochetes in smears. When received by the laboratory, June 19, no nodular or scaly lesions were apparent on the vulvar surface, but smears of serum obtained by scarifying the slightly reddish mucous surface showed a few spirochetes. This lesion showed spirochetes 3 weeks later; and a urine specimen, not catheterized, showed 1 spirochete, the presence of which was explained by the discovery of a condylomatous lesion in the vagina which discharged serum loaded with spirochetes. On July 3, spirochetes were demonstrated in blood smears. The rabbit was killed on August 18; the tissues of the lesion showed spirochetes, but the organs were negative both as to lesions and the presence of spirochetes.

Rabbit B.—A small female was procured by Dr. A. C. Starry and inoculated from rabbit A on the vulva and anus, about May 8. Spirochetes showed in the lesion about June 5, and when received by us, June 19, showed only slight nodules at these points, which, however, when scarified and smeared showed many spirochetes. On October 9, a scab the size of a wheat kernel was discovered hanging from the lip. The freely bleeding surface under the scab showed many spirochetes. The anal and the vulvar lesions, perhaps because of frequent scarification for subinoculations, are still large, papular, scaly and bleed easily. No spirochetes were found in the blood.

Rabbit C.—A large brown male was inoculated on June 22 from rabbit B on scarification at the upper penile margin. This appeared slightly red on July 10, and was scarified and smeared, showing spirochetes. The rabbit had a pyogenic testicular infection when procured which did not clear up and which resulted in death on July 31. The tissues of the lesion showed numerous spirochetes; the organs were negative, both as to pathologic changes and spirochetes.

Rabbit D.—A small brown female was inoculated from rabbit B on June 27 and on July 6 showed a pinkish spot at the site of scarification, which disappeared by July 8; but smears from the scarification at the spot on July 10 showed spirochetes. The lesion developed typically, becoming papular with an eroded scaly surface, bleeding easily, and was active when the rabbit was killed on Nov. 15. The tissues showed no spirochetes except in the lesion; and no pathologic changes elsewhere.

Rabbit E.—A large albino female was inoculated intravenously on Aug. 18 with 2 cc. heart's blood from rabbit A at necropsy, but no lesions developed, and the rabbit remained in good health. On Oct. 26, a genital scarification was inoculated from the lesion of rabbit O, and a mild, slightly scaly lesion was discovered on December 4, which showed spirochetes. Blood smear, Dec. 15, showed spirochetes.

Rabbit F.—A small black female was inoculated with scarifying, from rabbit B, July 20, with no development of a lesion and no spirochetes showing on smears following scarification of the site of inoculation. A second inoculation, this time without abrasion of the tissues, was made from the lesion of rabbit O, which resulted in a lesion by Nov. 6, and is now eroded and scaly, extending around vulva to anal region.

Rabbit G.—A black and white male was inoculated on July 19 from rabbit B, but no lesion developed and no spirochetes could be demonstrated at the site of scarification. On Oct. 19, a second inoculation without abrasion was made from the lesion of rabbit O, the material being placed on the under side of the tip of the penis. The resulting lesion, atypically small, and lacking scaliness because of the moist surface, was found on Dec. 4 to be discharging many spirochetes, and remains atypical, except for ready bleeding.

Rabbit H.—A dark brown and white female failed to become infected from inoculation from rabbit B, July 19; but inoculation without scarification from O on Oct. 9 resulted in a lesion, now large and eroded, which gives smears rich in spirochetes.

Rabbit I.—A gray and white male developed a lesion from inoculation on July 20 from rabbit B. This lesion remained active, and when the animal was killed on Nov. 15, the lesion tissues showed spirochetes; the other tissues were negative, both as to the occurrence of pathologic changes and the presence of spirochetes.

Rabbit J.—This rabbit was inoculated from rabbit B on July 20. On Aug. 16, there was a small eroded lesion on the upper margin of the penis in which spirochetes were found on scarification. The lesion increased in extent after scarification and was covered with scales. On removing the scales, the area bled easily, a characteristic finding in all the cuniculi lesions. When placed in a breeding pen with rabbit R, Oct. 21, J still had an active scaly lesion, containing numerous spirochetes. Now the lesion is involuting, has no scales and slight redness. No spirochetes were found in the blood stream.

Rabbit K.—This rabbit was inoculated from rabbit O without scarification on Oct. 9. After 56 days, a slight reddened area appeared above the penis, slightly eroded. Smears from this showed a few spirochetes. At present the lesion is much the same with a slight scaliness. No spirochetes have been found in the blood stream.

Rabbit L.—This rabbit was inoculated from rabbit D on Aug. 10. After inoculation, L lost weight rapidly, and appeared to be suffering from coccidiosis. On Sept. 1, spirochetes were found in a small eroded lesion on the vulva. Later the lesion became more extensive, bleeding easily, and was covered with scales. The rabbit died on Sept. 9. Spirochetes were found in the tissue of the lesion, with the silver-agar stain, but none in the other tissues; and no spirochetal lesions were found elsewhere.

Rabbit M.—This rabbit was inoculated, Aug. 10, from rabbit D. On Aug. 24, numerous spirochetes were found in a reddened eroded area on the vulva. This lesion was almost similar to the others, increasing in extent, shallow erosions covered with scales and bleeding freely on removal of the scales. On Sept. 9, one spirochete was found in a blood smear. Later smears showed no spirochetes. The rabbit was killed on Nov. 15. At this time the lesion had involuted slightly. With the silver-agar method, spirochetes were stained in the tissue of the lesion but not in the other tissues. No pathologic lesions were found elsewhere.

Rabbit N.—This rabbit was inoculated from rabbit M on Sept. 13. On Sept. 20, spirochetes were found in a small eroded genital lesion. This later became extensive, bleeding on slight manipulation. Blood smears taken on Oct. 10 showed one spirochete. Numerous other blood smears showed none. This lesion is still extensive and active, yielding smears rich in spirochetes.

Rabbit O.—This rabbit was inoculated from rabbit M on Sept. 13. Spirochetes were found on Sept. 25 in an erosion on the vulva. Later this lesion spread over the entire genital region and now involves the external genitalia and also 2 small areas around the anus. Smears yielded great numbers of spirochetes.

Rabbit P.—This rabbit was inoculated on Oct. 5 from rabbit O, without scarification. Negative results were obtained. On Oct. 26, it was inoculated from rabbit N. On Oct. 31, spirochetes were found in an unpromising looking reddened area on the upper margin of the penis. The lesion increased somewhat in extent. At present it shows a small scaly lesion on each side of the upper margin of the penis, slightly involution. A spirochete was found in a blood smear taken on Nov. 10.

Rabbit Q.—This rabbit was inoculated without scarification from O on Oct. 5, with negative results. On Oct. 26, it was inoculated from rabbit N. Spirochetes were found in a small erosion in the genital region on Oct. 31. The lesion has never been extensive, and is now scaly, with slight redness and involution. A spirochete was found in a blood smear taken on Nov. 10. All other blood smears were negative.

Rabbit R.—This rabbit was placed in a breeding pen with rabbit J on Oct. 21. Slight redness and erosion of the genitals were found on Dec. 4. Smears from this showed spirochetes. Blood smears taken on Dec. 15, showed a single spirochete. The lesion involves a large part of the external genitals but shows less scaling than most lesions in the female rabbits.

INCUBATION TIME

The time required for the development of the inoculated lesion varied from 5 to 56 days, the average time for 16 inoculations being 23.81 days. The inoculations without scarification or with little scarification developed most slowly.

COURSE OF THE INFECTION

The local lesions varied from slight macular or papular, scaly or eroded lesions to more severe condylomatous lesions with abundant exudate. In no case did bubo develop; and in none were any symptoms of a generalized infection discoverable. The rabbits remained in good health, except for intercurrent pyogenic infection and coccidiosis in 2 animals dying spontaneously. In one case alone did a lesion containing spirochetes develop in another part of the body; this occurred as an autoinoculation of the lip in rabbit B, 5 months after the primary genital

inoculation; the anal and genital lesions in this rabbit were at this time very active. In some cases the lesion remained local, being confined to the point of inoculation, and in such cases the infection was very mild; in the other cases, it spread by extension and contiguity over the entire genital and anal mucosa, and involved the neighboring skin to some degree. Even in the most severe condylomatous forms, no enlargement of the neighboring glands was noted. The severity of the lesions seemed to bear some relation in part to the individual resistance of the rabbit, but especially to the degree of local mechanical irritation and trauma. Mild lesions left unirritated showed a marked tendency to involute and to become latent. Such lesions could be excited into active ones by scarification or mechanical irritation. The importance of local trauma is great, both as to securing a successful inoculation and as to the activity of the course of the infection. No spontaneous absolute cure took place in any of our cases, due possibly to the fact that the lesions were frequently irritated for the purpose of securing spirochetes. Even when the lesion seemed practically healed or very mild, spirochetes could still be obtained by scarification when they could not be obtained from the surface. In the case of the active lesions, however, surface smears always yielded great numbers of spirochetes. In general, it appears that the lesions tend to involute spontaneously if not irritated, and that a relative local immunity may be obtained, the spirochetes persisting in a mild chronic or latent lesion. This relative local immunity is easily upset by trauma, even when slight. There is no general immunity, even after 5 to 6 months. Reinfection through inoculation may be produced by scarification in any part of the skin or mucous membranes during this time.

PRESENCE OF SPIROCHETES IN THE BLOOD STREAM

Spirochetes were demonstrated in blood smears made from the general circulation, obtained from the ear and under conditions preventing any contamination with spirochetes on the skin or mucous membranes. A positive demonstration of their presence in the circulating blood was obtained in 6 cases. This was accomplished only after an extensive search in each case; the organisms occurred singly in the positive smears; only well defined and preserved organisms were considered as positive spirochetes, and the amount of investigation necessary to demonstrate these and the few instances in which they were found proves

TABLE 1
STUDIES IN SPIROCHETA CUNICULI

Rabbit	Inoculated		Positive		Consecutive Transmission	Blood Examined for Spirochetes	Termination to Date
	Date	From	Date	Day			
A* ♀	1	7/3+ 7/6— 7/11— 7/24— 8/17—	Killed 8/18; lesion positive; other tissues negative
B ♀	5/ 8	A	6/ 5	28	2	7/5— 7/6— 7/11— 7/24— 8/17— 9/15— 10/20— 10/27— Wassermann Neg. 1/5/23	New lesion on lip 10/9; this and anal and genital lesions active
C ♂	6/22	B	7/10	18	3	7/24—	Died 7/31 of pyogenic infection; lesion showed spirochetes; other tissues neg.
D ♀	6/27	B	7/10	13	3	7/24— 8/17— 9/18—	Killed 11/15; lesion showed spirochetes; other tissues negative; some coccidiosis present
E ♀	8/18† 10/26	A 0 12/ 4	.. 39	... 6 12/15+ Wassermann Neg. 1/5/23	Eroded lesion, little scabiness, mild
F ♀	7/20 10/ 9‡	B 0 11/ 6	.. 27	... 6 11/7— 11/10— 11/14—	Lesions around vulva and anus extensive
G ♂	7/19 10/ 9‡	B 0 12/ 4	.. 56	... 6	12/15— Wassermann± 1/5/23	Small eroded lesion under side of tip of penis discharging many spirochetes
H ♀	7/19 10/ 9‡	B 0	11/ 4	28	6	11/7— 11/10+ 11/14— Wassermann Neg. 1/5/23	Genital lesion active
I ♂	7/20	B	8/19	20	3	9/18—	Killed 11/15; lesion showed spirochetes; tissues neg.
J ♂	7/20	B	8/16	27	3	9/15— Wassermann Neg. 1/5/23	In breeding pen with R, 10/21; infection transmitted; lesion involuting
K ♂	7/20 10/ 9‡	B 0	12/ 4	56	6	12/15—	Lesion mild
L ♀	8/10	D	9/ 1	21	4	Died 9/14; severe coccidial infection found; spirochetes in lesion; none in other tissues
M ♀	8/10	D	8/24	14	4	9/18+ 10/5—	Killed 11/15; lesion positive; other tissues negative
N ♀	9/13	M	9/20	7	5	10/5+ 10/20— 10/26—	Lesion active
O ♀	9/13	M	9/25	12	5	10/5— 10/20— 10/27—	Large active lesion on genitalia; two small new ones around anus
P ♂	10/ 5‡ 10/26	0 N 10/31	.. 5	... 6 11/7— 11/10+ 11/14—	Scaly lesions on each side of upper margin of penis slightly involuting
Q ♂	10/ 5‡ 10/26	0 N 10/31	.. 5	... 6 11/7— 11/10+ 11/14—	Scaly genital lesion, sub-acute
R§ ♀	12/ 4	?	4	12/15+ Wassermann Neg. 1/5/23	Genital lesion active

* Spontaneous lesion found about February.
† Intravenous inoculation with 2 c.c. of blood.
‡ Inoculation without scarification.
§ In breeding pen with J, 10/21.

conclusively that their presence in the blood stream is incidental, and that there is no true spirochetemia in this infection. In addition to the few well-preserved spirochetes obtained from the blood stream, only a few broken degenerating forms were seen. We are confident from the amount of research given to this line of investigation that the entrance of the organisms into the blood stream in cuniculi infection is accidental and of rare occurrence.

As to the occurrence of spirocheturia in this infection, it is impossible to obtain urine from rabbits with genital infections without contamination from the lesions. Spirochetes were found in the urine of such rabbits obtained in various ways, but only in such numbers as to warrant the conclusion that it was due to contamination. This belief was confirmed by the fact that no spirochetes could be demonstrated in the kidneys obtained at necropsy, or in the bladder urine. Our work so far tends to show that this form of spirochetal infection differs from syphilis, infective jaundice and leptospiral infections in the absence of spirochetemia and spirocheturia.

Demonstration of Spirochaeta Cuniculi.—We prefer cover-glass smears stained by Warthin-Starry¹² silver-agar method, after fixing the dried smears for 3 to 5 minutes in absolute alcohol. The cuniculi organisms stain easily and beautifully an intense black by this method. The morphologic characteristics of the spirochete can be studied in such stained smears much more precisely than in the dark field, and the danger of confusing cuniculi with pallida in such preparations seems to us to be very slight. In the tissue lesions, however, cuniculi do not take the silver impregnation as readily or as intensely as pallida. Nevertheless, we have obtained good results by altering our silver-agar method for the staining of tissue sections on cover-glasses, by treating the sections for a longer period with H_2O_2 , 1 to 24 hours. Without this treatment, the rabbit tissue turns a deep red-brown as soon as it is placed in the developing solution, and the spirochetes cannot be seen for lack of contrast. It was also found that 2 drops of hydroquinone solution gave better results than 1 c.c. as advised for the staining of pallida. One splendid result was obtained by soaking the tissue section in a buffer solution for 2 days and then for 20 minutes in H_2O_2 , but for unknown reasons this result could not be duplicated. The difficulty in applying the silver method to sections of rabbit tissue seems to lie

¹² Jour. Infect. Dis., 1922, 30, p. 592.

rather in the peculiar reaction of the tissue elements to the staining method than in the spirochetes themselves. It is impossible to get as sharp and clear a contrast between the tissue elements and the spirochetes as can be accomplished between pallida and human tissue elements. Nevertheless, they can be demonstrated by the Warthin-Starry method in a satisfactory manner for diagnostic purposes, but with somewhat less satisfactory results than in the case of pallida in human tissue. The cuniculi spirochetes, further, are found chiefly on the surface of the lesion and in the epithelium or just below it, where they form large crowded and entangled masses from which it is difficult to focus out all the parts of a single organism. They rarely occur in the tissues as single organisms, as is the frequent case with pallida.

Characteristics of Spirochaeta Cuniculi.—In the silver preparations on cover-glasses, they resemble morphologically some of the mouth spirochetes much more closely than they do pallida. They are thicker and larger, as a rule, than pallida; they are softer and more flexible and easily straightened out in smears. They have a marked tendency to form circles, V and Y shapes, and twisted stellate forms. Many of them are somewhat straightened out in the middle of their length, and the winding beyond this straight area often seems to be in a reverse direction, giving the appearance of 2 organisms with turns winding in opposite directions attached to each other at one end. Often 2 organisms are twisted together at one end, the other ends widely divergent. When bent in the middle the bend is always curved; the sharp right angle bending that is so characteristic of pallida does not occur.

The measuring of 1,000 cuniculi organisms gave the following results:

Average length, 11-12 μ .	Amplitude, 1.0-1.2 μ .
Average number of turns, 8-9.	Spiral depth, 0.6-0.8 μ .
Length variations, 6-21 μ .	Width, 0.2 μ .
Variation in turns, 6-17.	

No terminal granules or flagellae are indicated in the silver stain, although with other forms of spirochetes they are shown by this method. When the ends are in sharp focus they appear blunt, and do not taper as in the case of pallida. When contrasted side by side in the silver stain, pallida and cuniculi are more easily differentiated than pallida and some of the mouth spirochetes. In specimens showing

the same length and number of turns, there is always an impression of stiffness and rigidity, and tightness of turns in pallida lacking in the other organism. To say that the cuniculi organisms are indistinguishable morphologically from pallida is an exaggeration as far as the appearances of these organisms in silver-agar cover-glass smears are concerned. To us the morphologic differentiation of these two spirochetes seems easier than in the case of pallida and some of the tightly coiled spirochetes occasionally found in the human mouth of nonsyphilitic persons. As for the morphologic differentiation in the dark-field, there can be little doubt that mistaken identifications could easily take place, just as mistaken dark-field identifications are being made frequently in the case of pallida and mouth and smegma spirochetes. In the tissues, the difficulty of differentiation between cuniculi and pallida is greater, and it would be much easier to make mistakes in the case of silver impregnations of tissue containing cuniculi. This applies, of course, to the identification of single organisms. If the whole picture is considered, the danger of mistaken identification of cuniculi in the tissues for pallida becomes greatly reduced. The superficial localization of cuniculi, the growth through the epidermis, the formation of thickly entangled confused masses of the organisms—an epithelial colonization rather than a vascular one—give a picture very different from that seen in pallida infections. The ease of differential identification will increase greatly with increased acquaintance with the cuniculi. At first they may seem to resemble pallida very closely morphologically, but on older acquaintance the similarity does not seem so close.

PATHOLOGY

Necropsy was performed on 6 cases of cuniculi infection; 2 of the cases were spontaneous deaths from other processes, the other 4 were killed at various stages of the lesion, acute, subacute and chronic. The chart shows the relative ages of the infection in the 6 cases. The pathologic changes will be presented here in a condensed form.

Local Lesion.—The local lesion shows the microscopic appearances of a chronic infective granuloma tending to assume a papillomatous or condylomatous character. In the earlier stages, the upper layer of the submucosa or corium is infiltrated with lymphocytes and polynuclears, and occasional eosinophils, forming a narrow inflammatory zone beneath the epithelium. Over this area, the epithelium is hyperplastic and shows more or less leukocytic infiltration. The blood vessels show more or less marked hyperemia, but there are no

perivascular infiltrations such as those in the pallida infections. The lesion histologically does not resemble the early stages of a chancre. The surface epithelium becomes thickened and desquamates in horny scales more or less adherent to the surface, which becomes covered with a scaly scab. On the surface of the epithelium, but particularly in the mucosa or epidermis, the spirochetes can be demonstrated in enormous numbers. Beneath the epidermis, they are rarely found at this stage at a greater depth than 1 mm. In more active lesions, the infiltration of the papillary layer or submucosa becomes more marked, the epithelium more hyperplastic and the interpapillary epithelial cords more marked and increased in length. On cross section, these appear as islands of hyperplastic epithelium often heavily infiltrated with eosinophils. The epithelium of the ducts of the glands and of the hair follicles takes on a similar hyperplasia and infiltration; in some cases, these interpapillary epithelial plugs are filled with pus cells. The inflammatory infiltration is always most marked around the epithelium, and not perivascular. There is no marked proliferation of blood vessels as in the chancre, and no perivascular mantling or endothelial proliferation as in syphilis. The vascular whorls so characteristic of syphilis are entirely wanting. At its height, the lesion assumes a typical papillomatous or condylomatous growth with infiltrations involving the upper layers only of the mucous membrane or skin. There is marked epithelial hyperplasia, as in the gonorrheal wart, which the lesion resembles much more than it does a syphilitic sore. In some areas the epithelial plugs are so infiltrated with eosinophils that they stain a deep red. Erosion or ulceration of the infiltrated area may take place, but the loss of tissue is superficial. The shallow defect is usually covered with a crust of exudate containing enormous numbers of the spirochetes. The entire lesion is a superficial one; in the most extensive form, the infiltration does not extend into the skin or subepithelial tissues for more than 3 to 5 mm.; the infiltration and enlargement proceed outward in the lengthening and broadening of the papillae and the hyperplasia of the interpapillary epithelial plugs, and of the epithelium of the gland ducts and hair follicles. The infiltration of the epithelium is polynuclear and eosinophilic, that of the connective tissues is more lymphocytic in type, although at the height of the lesion many polynuclear forms and eosinophils are found near the epithelium. In the subacute and chronic stages the polynuclear infiltration is less marked, plasma-cells and fibroblasts appear in the papillae, the interpapillary epithelium becomes more horny and shows fewer wandering cells. The blood vessels do not show any obliterative proliferation and are but little thickened. In the nearly healed areas, the papillae are contracted, plump, or disappear; the epithelium, usually quite horny, is stretched over a narrow zone of scar tissue, in which islands of hyperplastic epithelium may still persist. In areas apparently completely healed, the only change is a narrow area of scar tissue beneath the epithelium. The whole process resembles closely the development and resolution of the condyloma acuminatum in man. No giant cells were found in any of the lesions.

In the sections of the local lesions stained by the silver-agar method for spirochetes, the cuniculi organisms are found in enormous numbers on the surface, in the epithelium and in the connective tissue just beneath the latter. They disappear rapidly in the deeper layers and do not occur in perivascular collections in the subcutaneous tissue as in the case of pallida in human

chancre. In the upper layers of the connective tissue, they may be found in the neighborhood of congested blood vessels, but their association is chiefly epithelial and not vascular.

The neighboring lymph nodes show congestion, edema and slight lymphadenitis. No direct involvement of these was noted.

General Pathology.—No lesions were found in any of the viscera in the 6 necropsy cases that in any way suggested syphilis. The internal organs were normal except in the 2 cases of spontaneous death due to coccidiosis and pyogenic infection. In these multiple inflammatory areas, beginning pyemic abscesses, were found in the kidney.

Sections of tissue from all of the internal organs stained by the silver-agar method were entirely negative as to the presence of spirochetes, either in the tissues or blood vessels. The pathologic data afforded by the study of these 6 cases gives no evidence supporting the theory that the cuniculi infection is in any way a syphilis-like disease. The results of our study up to the present time indicate that this infection is a local chronic infective granuloma due to the localized growth of spirochetes, and not a generalized spirochetosis.

WASSERMANN REACTION

The Wassermann reaction was found to be negative in Rabbits B, E, H, J, and R. In Rabbit G, a doubtful result (\pm) was obtained, but no significance is attached to this. The heart blood was used. The antigen employed was a cholesterinized alcoholic beef heart extract, and water bath incubation results were checked by reading after refrigeration.

CONCLUSIONS

The study of 18 rabbits infected with *Spirochaeta cuniculi* shows that the spirochetosis produced by this organism is a superficial lesion, papillomatous or condylomatous in character, limited to the mucous membranes or skin, and spreading by continuity or contiguity or auto-inoculation, and transmissible by inoculation, contact and coitus. The lesions are essentially epithelial and not vascular, limited to the epithelial surface and upper portion of the subepithelial tissues, with marked hyperplasia of the epithelium and papillary layer. A well-developed lesion is essentially a condyloma, and does not suggest a chancre histologically. No evidence of systemic infection was found. No lesions containing the spirochetes were found in any of the internal organs. The general health of the infected animals remained good except in the case of intercurrent infections. The Wassermann reaction was negative. No general immunity was developed. The local lesions, when protected from trauma or local irritation, showed a tendency to resolve and become slight scaly latent lesions, from which spirochetes could still

be obtained in numbers by scarification, and which, through trauma, could be revived into more active and extensive lesions.

From the lesions the spirochetes can be easily obtained by cover-glass smears of the surface, or by scarification. The organism is most easily and best demonstrated in cover-glass smears stained by the Warthin-Starry silver-agar method for cover-glass smears. In such preparations, its morphologic characteristics are more clearly seen than they can be in the dark-field or by other staining methods; and to one acquainted with both forms there should be no danger of confusing cuniculi with pallida. In sections of the tissue lesions, the spirochetes may also be easily stained by the Warthin-Starry silver-agar method, but they are less satisfactorily demonstrated in rabbit tissues than is pallida in human tissues. In the tissue, cuniculi lie in thickly crowded and entangled masses on and in the epithelium, and just below it. They are not found in numbers very deep in the tissue. Their localization is essentially an epithelial one, and not a vascular one. Their entrance into the blood stream is accidental and occasional. No generalized spirochetemia or spirochetosis occurs in this infection. No colonization of cuniculi in any of the internal organs was found. Because of the genital lesions, urine and semen are easily contaminated; but no evidence of any true spirocheturia is as yet forthcoming.

Cuniculi spirochetosis is, therefore, a local disease of epithelial surfaces, the clinical course and local and general pathology of which do not in any way resemble those of syphilis. It should not be called "rabbit syphilis," and the term paralues cuniculi should also be discarded. The spontaneous rabbit disease can be easily differentiated from pallida infections by its morphology, as shown in silver-agar cover-glass smears, and by the pathology of the lesions.

Nevertheless, it is disconcerting to find that there is a spirochete infection of rabbits, apparently widespread throughout the world, caused by a spirochete that resembles pallida sufficiently closely to make possible the occurrence of mistaken identifications of the one for the other, in the case of any worker who is not acquainted with the two organisms and their differential diagnosis. It is unfortunately true that the great mass of experimental work on the transmission of human syphilis to the rabbit has been carried out in such ignorance of the spontaneous rabbit disease, and the value of that work now becomes legitimately doubtful. It is extremely likely that mistakes have been made. Now

that the knowledge of this primary rabbit spirochetosis has been obtained, the possibility of future error should disappear with intimate acquaintance with the cuniculi organism. It will, however, be necessary to repeat much of the work in this new light, particularly that concerned with the production of rabbit syphilis from parietic brains and human semen, and the production of reinfection and superinfection, immunity and cure. Too many important deductions have been made from rabbit experimental work to admit of any doubt being allowed to remain as to their accuracy.

PLATE 1

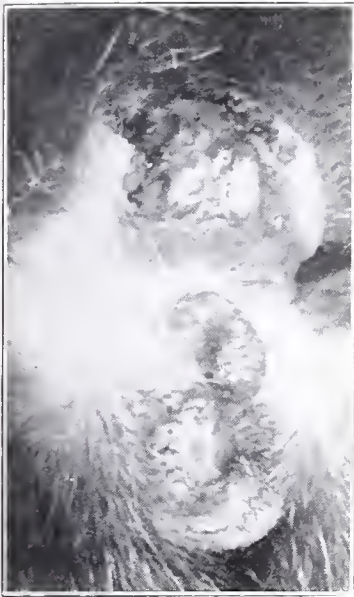


Fig. 1.—Rabbit O, genital lesions at full development, 3 months' duration. Fifth generation of inoculation; inoculated from Rabbit M.



Fig 3.—Rabbit B, auto-inoculated lip lesion, two weeks' duration.

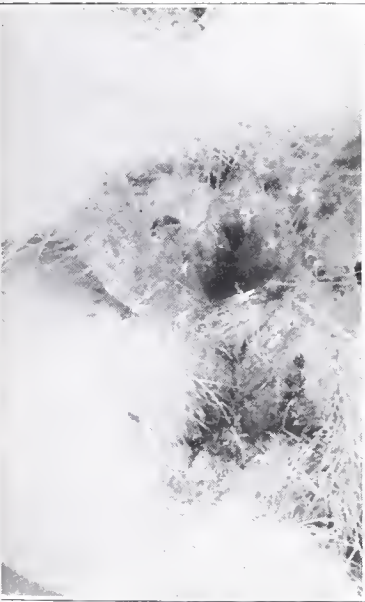


Fig. 2.—Rabbit B, anal and vulvar lesions at 3 months. Inoculated from A.



Fig. 4.—Rabbit A, condylomatous lesion of 4 months' duration; spontaneous lesion.

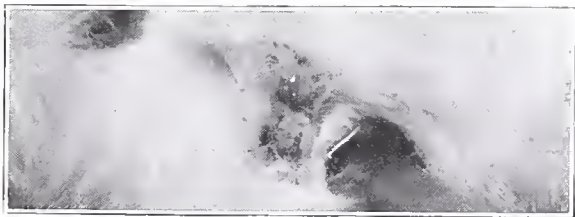
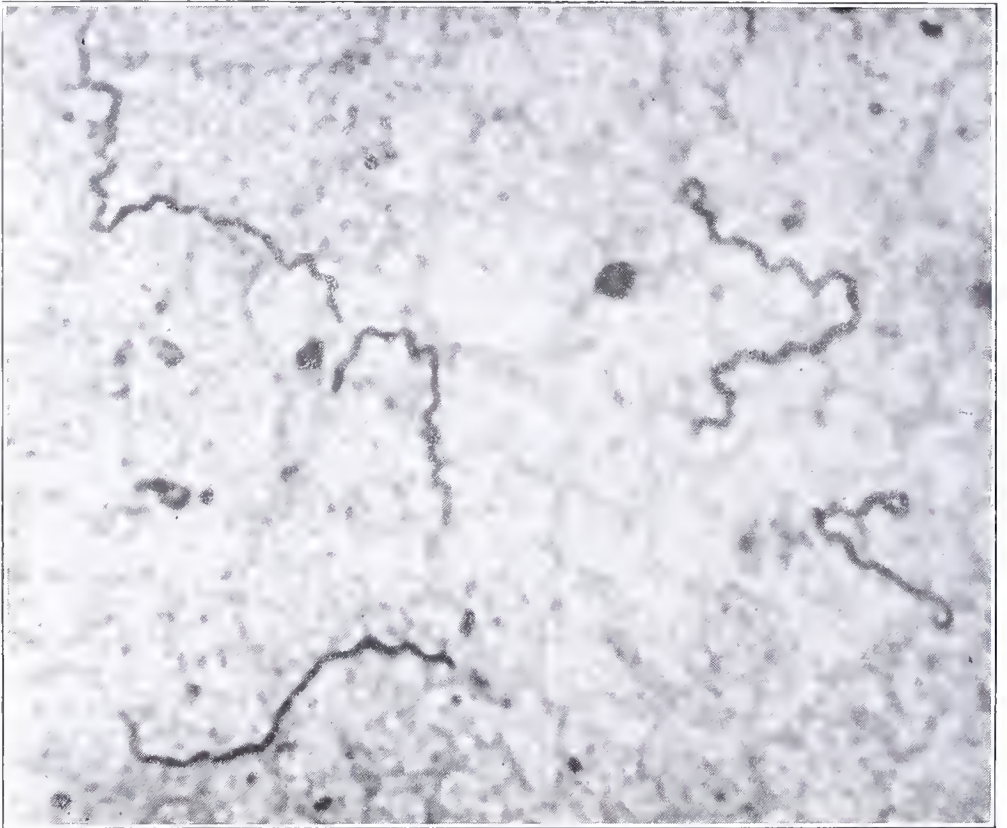
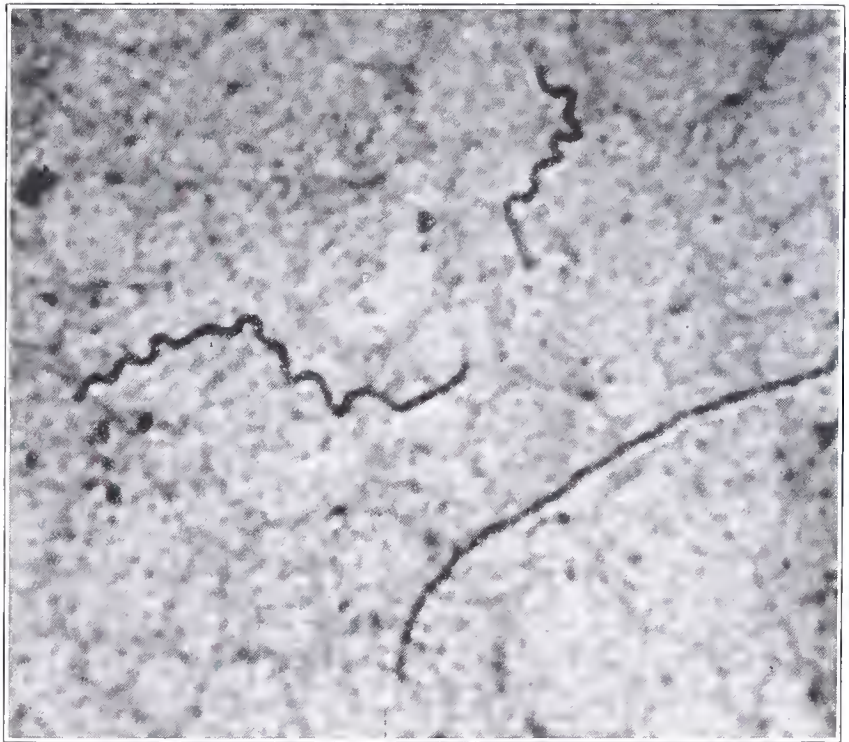


Fig. 5.—Rabbit D, lesion at 2 months; inoculated from B.

PLATE 2



A



B

Fig. 6. *A*, smear from scarified lesion of rabbit A, stained by Warthin-Starry silver-agar cover-glass method. *B*, the straight form produced by pressure on the cover-glass in making the smear. Photomicrograph, B. & L. oil immersion 2 mm., compensatory ocular No. 4, bellows length 155 cm.

PLATE 3

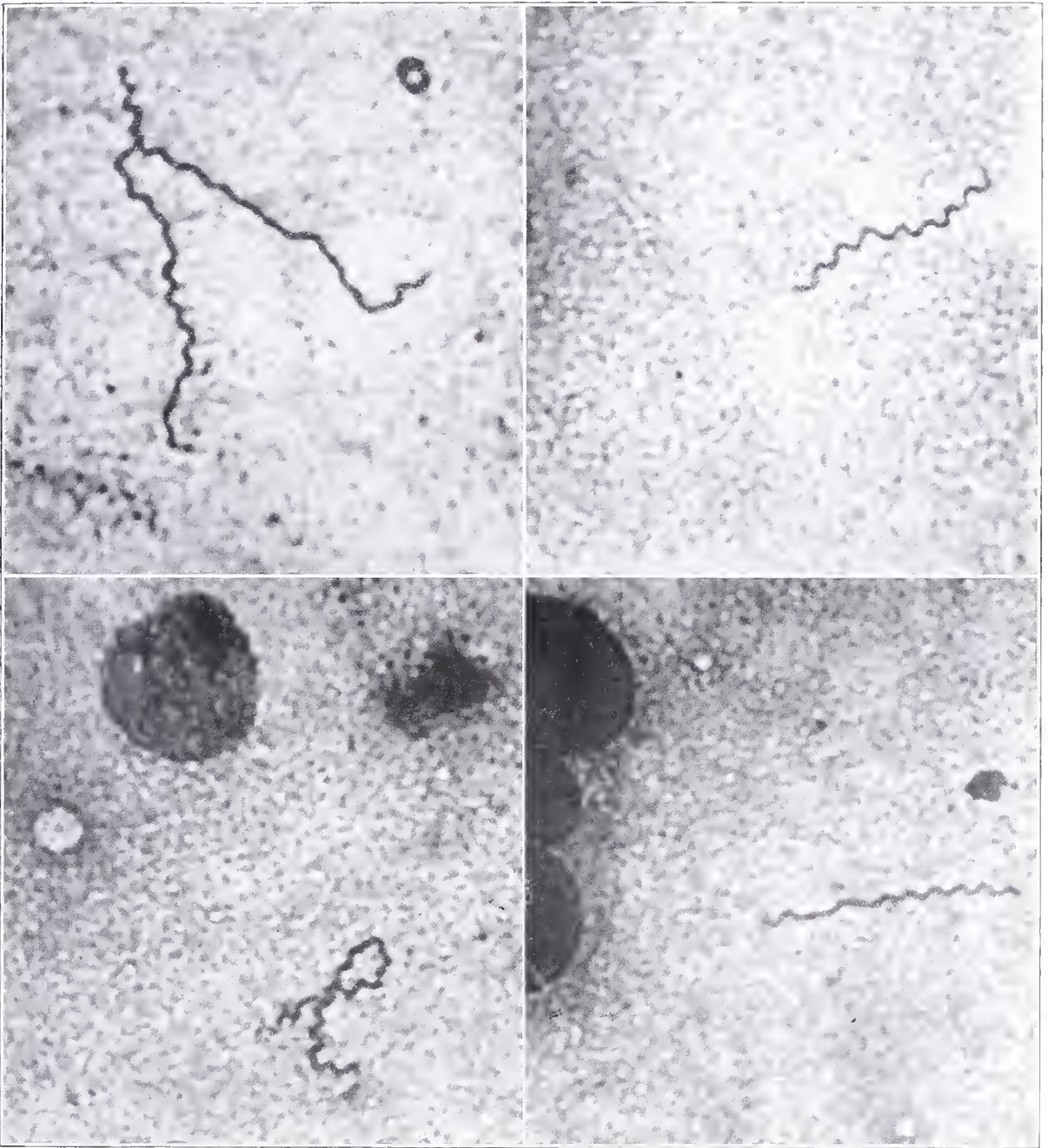
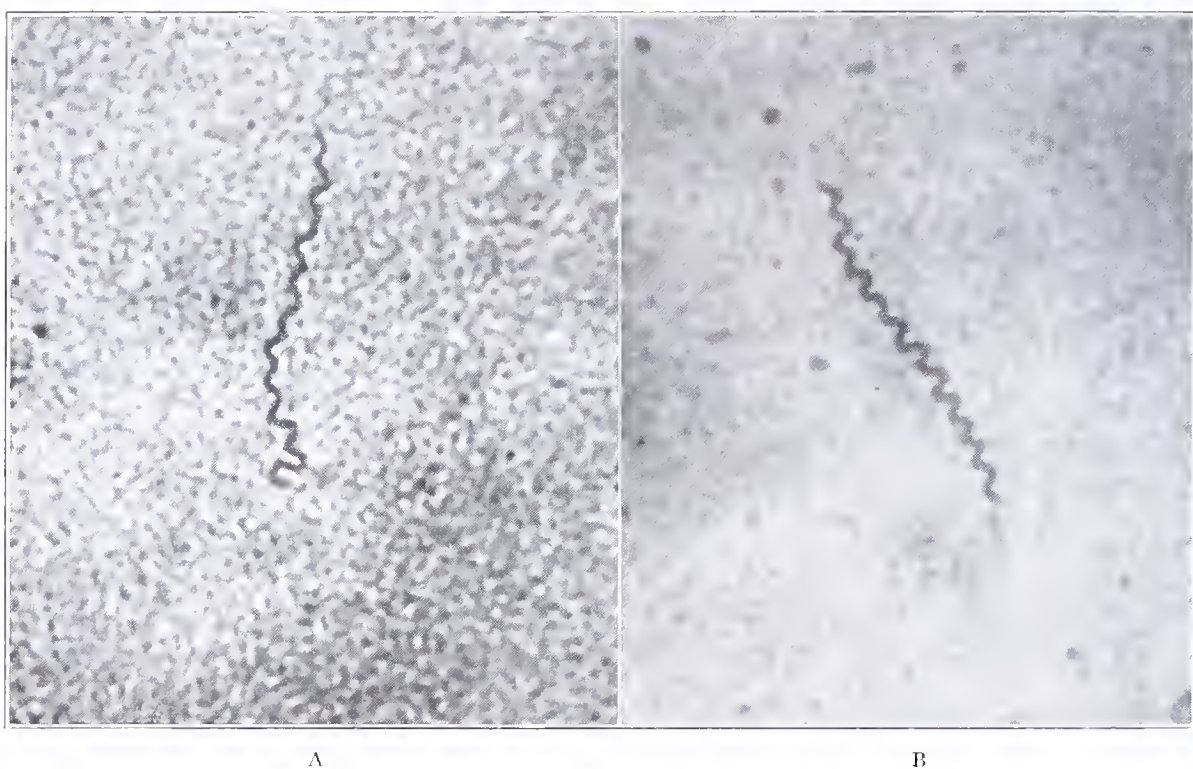


Fig. 7.—Characteristic morphology of *Sp. cuniculi* in cover-glass smears from rabbit venereal lesions, stained by the Warthin-Starry silver-agar cover-glass method. Photomicrographs, B. & L. oil immersion 2 mm., compensatory ocular No. 4, bellows length 155 cm.

PLATE 4



A

B

Fig. 8.—*A*, *Spirochaeta cuniculi* in cover-glass smear from rabbit venereal lesion. *B*, *Spirochaeta pallida* in cover-glass smear from human chancre. Both stained by Warthin-Starry silver-agar cover-glass method. Photomicrographs, B. & L. oil immersion 2 mm., compensatory ocular No. 4, bellows length 155 cm.

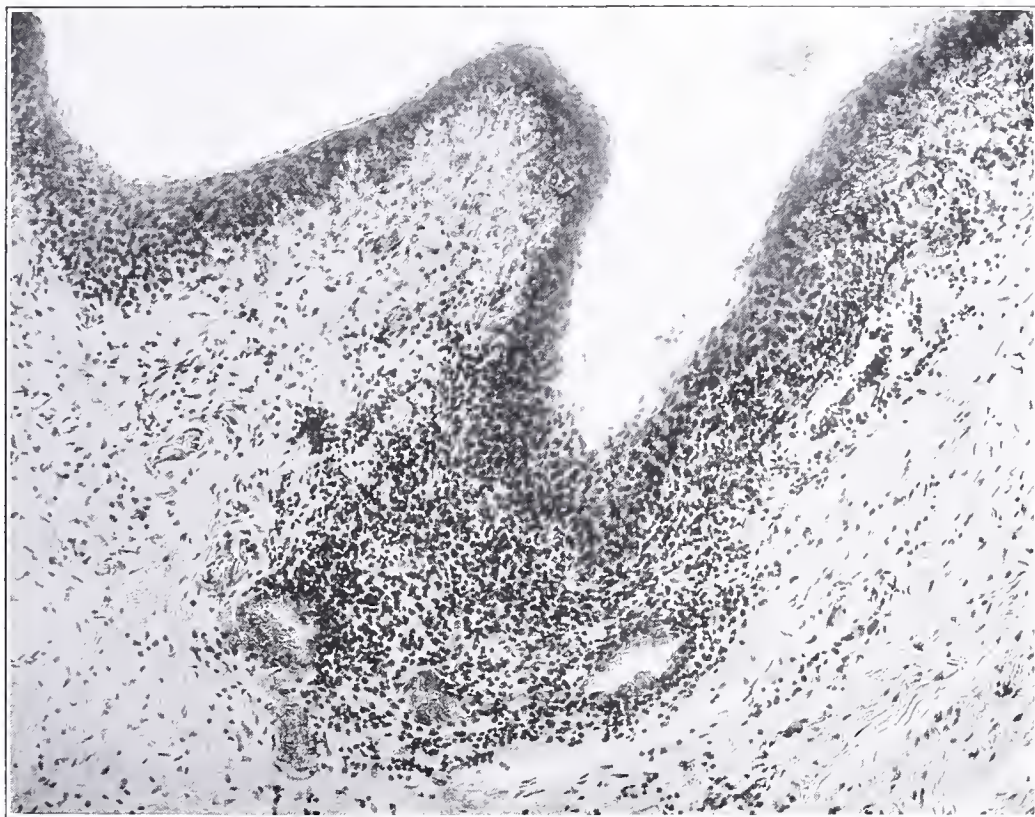


Fig. 9.—Histologic appearances of cuniculi lesion 2 weeks old. Scarification yielded great numbers of spirochetes, and the silver stain showed the tissue loaded with them. Low-power magnification.

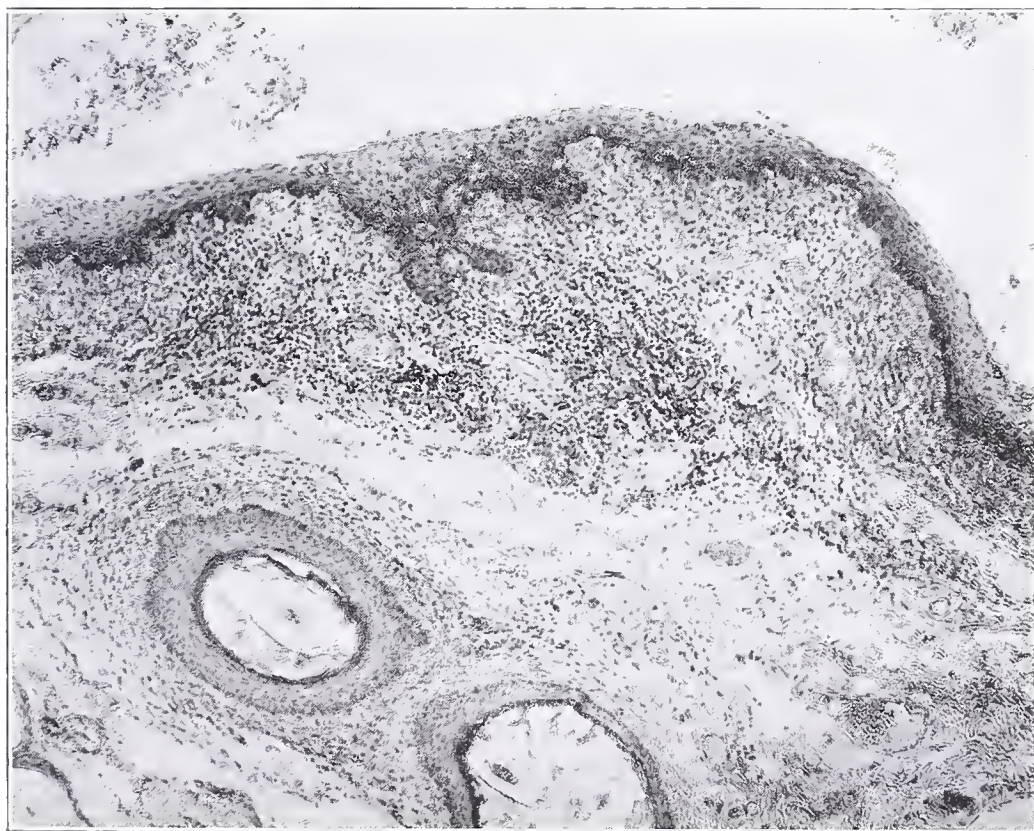


Fig. 10.—Low-power view of macular cuniculi lesion 2 weeks old. This lesion was full of spirochetes.

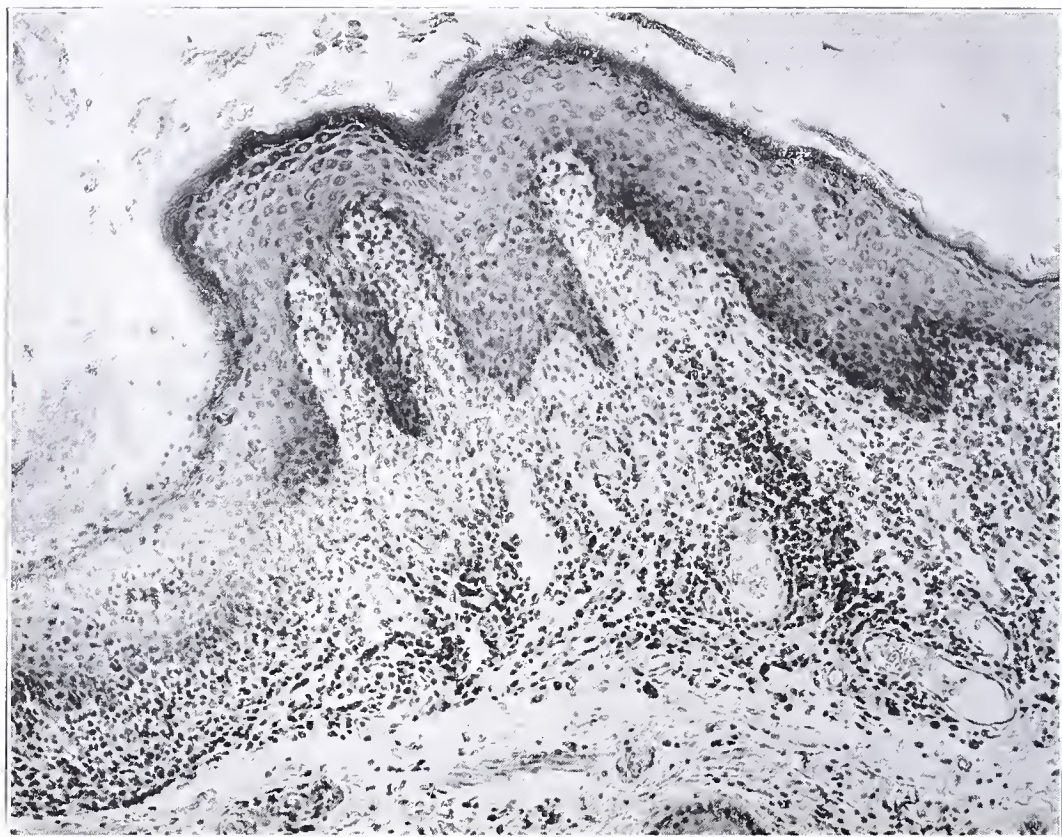


Fig. 11.—Low-power view of scaly cutaneous lesion 2 months old. Silver stain showed epidermis and upper layer of corium full of spirochetes.

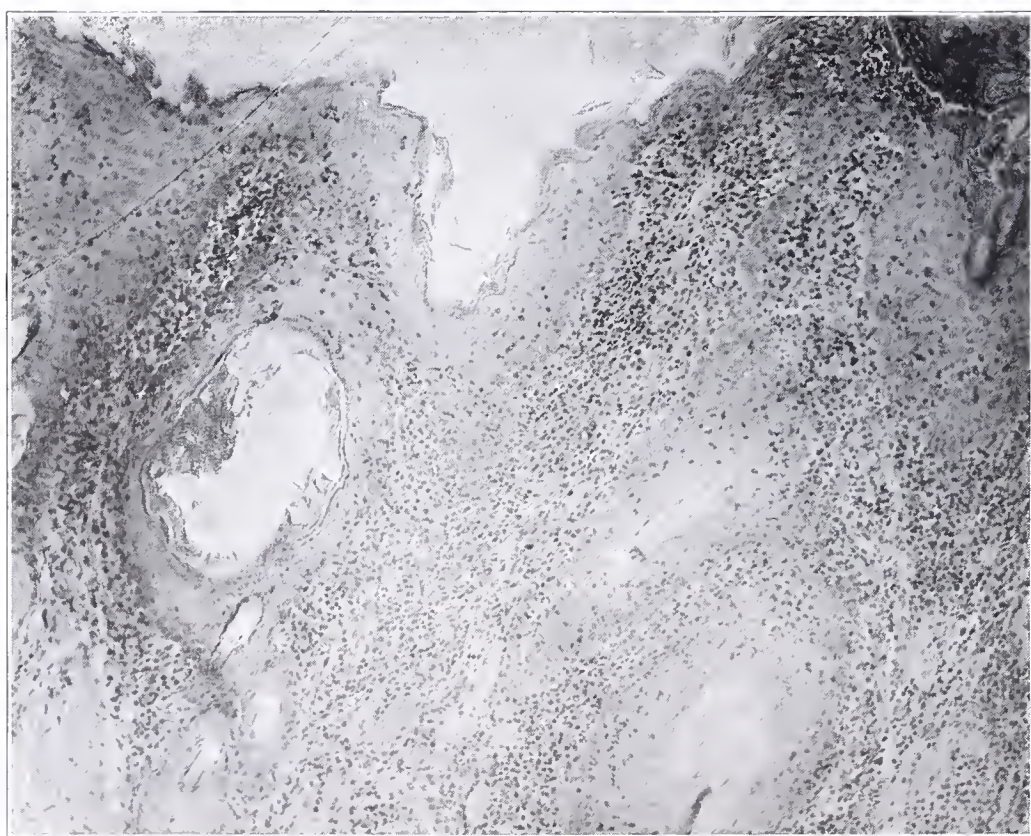


Fig. 12.—Surface of fully developed condylomatous lesion 3 months old.

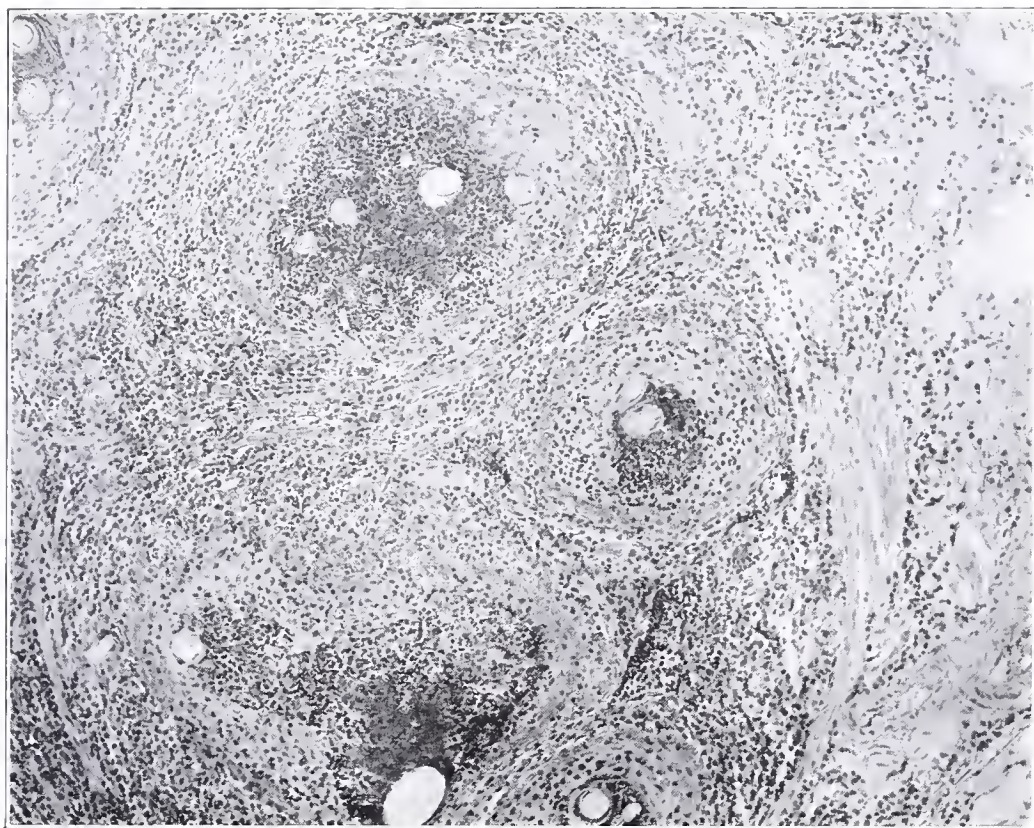


Fig. 13.—Same lesion at middle level.

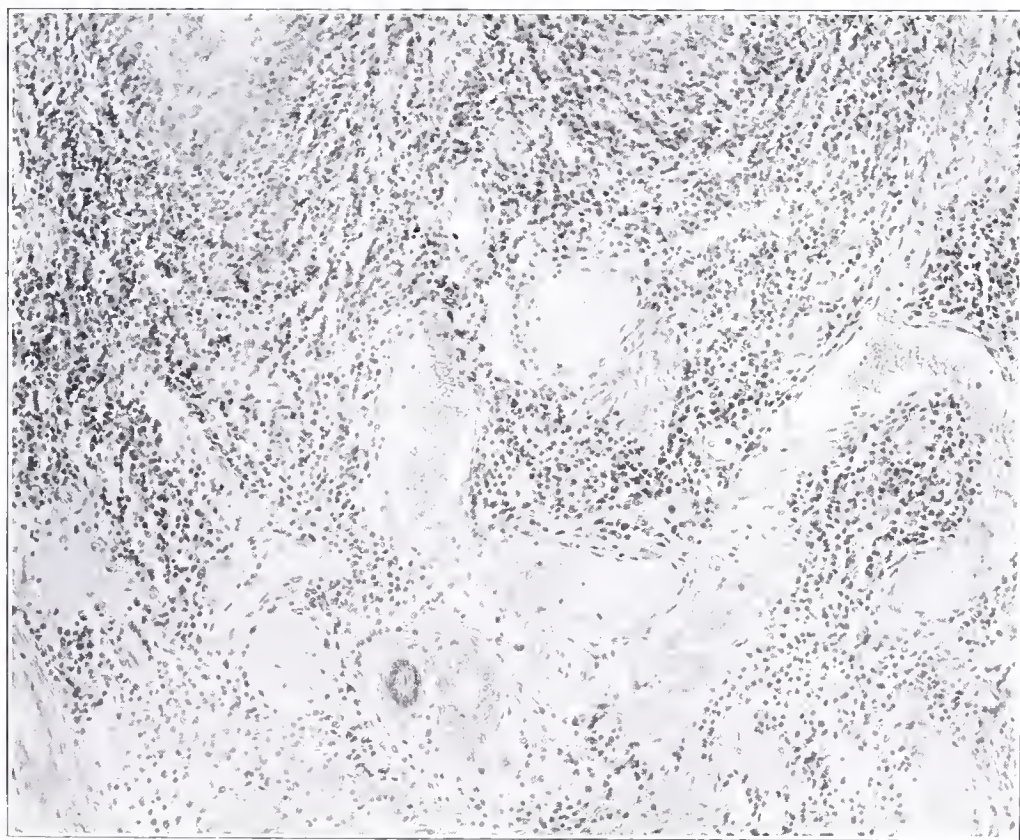


Fig. 14.—Same lesion at bottom level.

PLATE 8



Fig. 15.—Lateral border of same lesion.

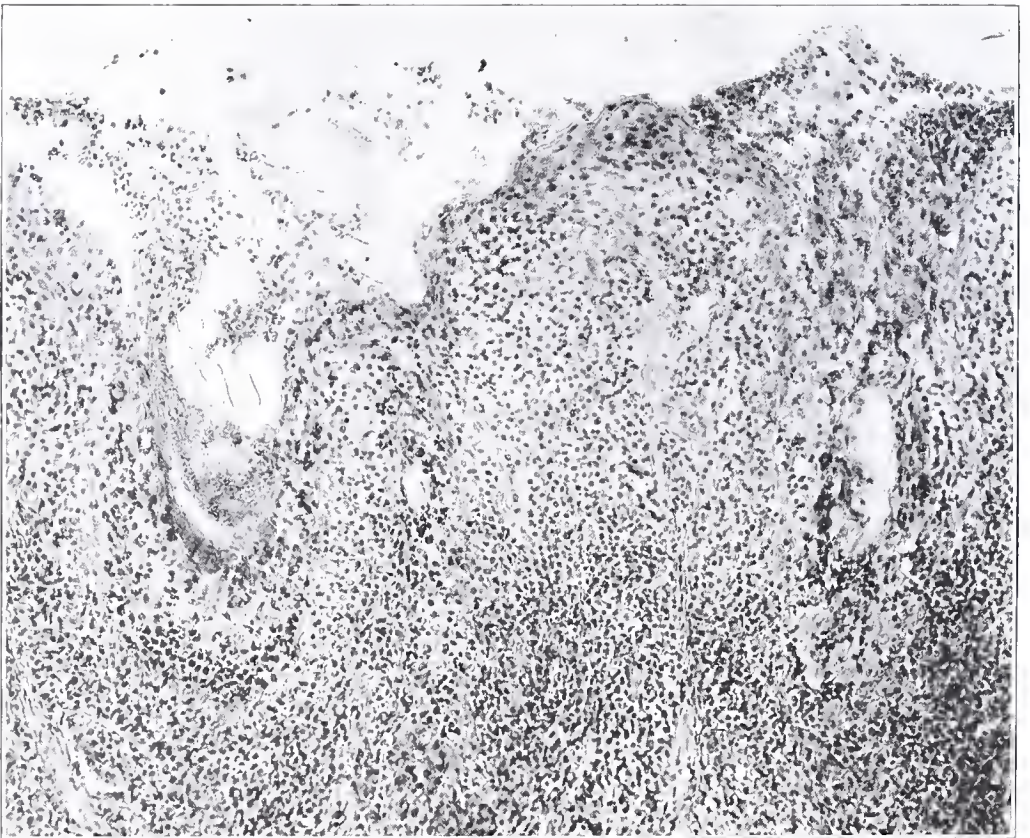


Fig. 16.—Area of beginning ulceration in genital lesion 3 months old. Upper layers were crowded thickly with spirochetes.



Fig. 17.—Genital cuniculi lesion in early stage of resolution; hyperplastic rudimentary lymph node. Spirochetes in great numbers still present.



Fig. 18.—Nearly healed condylomatous cuniculi lesion 4 months old. It still contained great numbers of spirochetes.

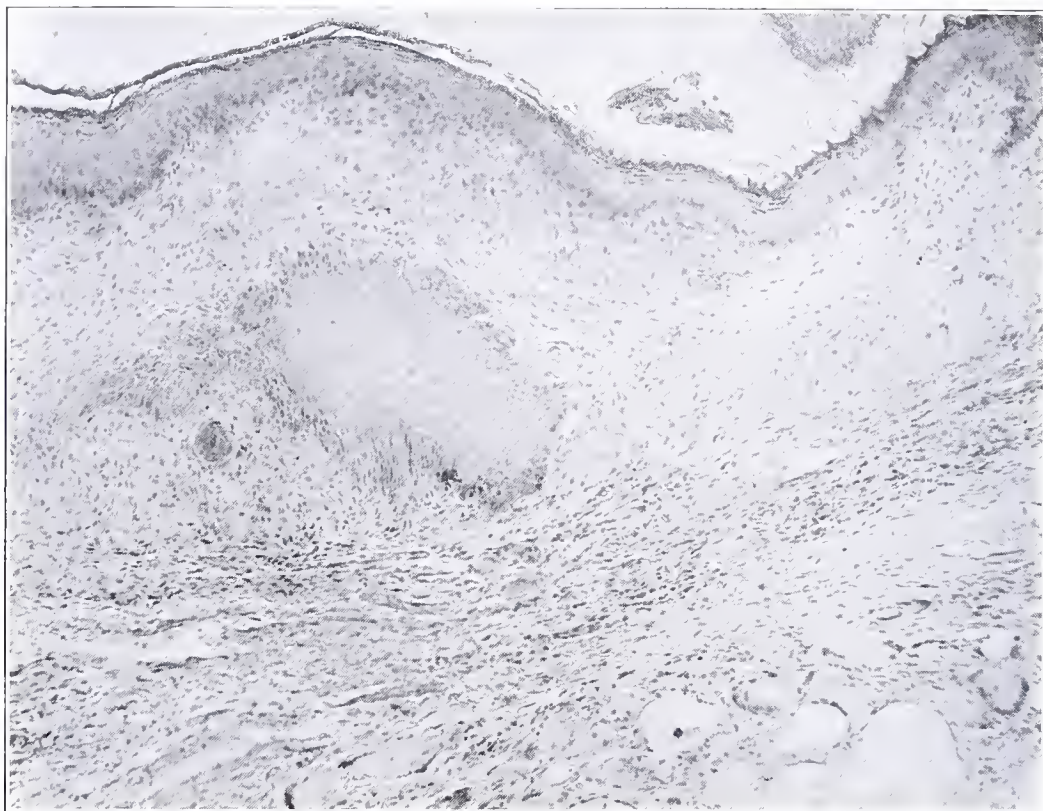


Fig. 19.—Nearly healed cuniculi lesion 4 months old. Numerous spirochetes were still present in upper layer and in epidermis.

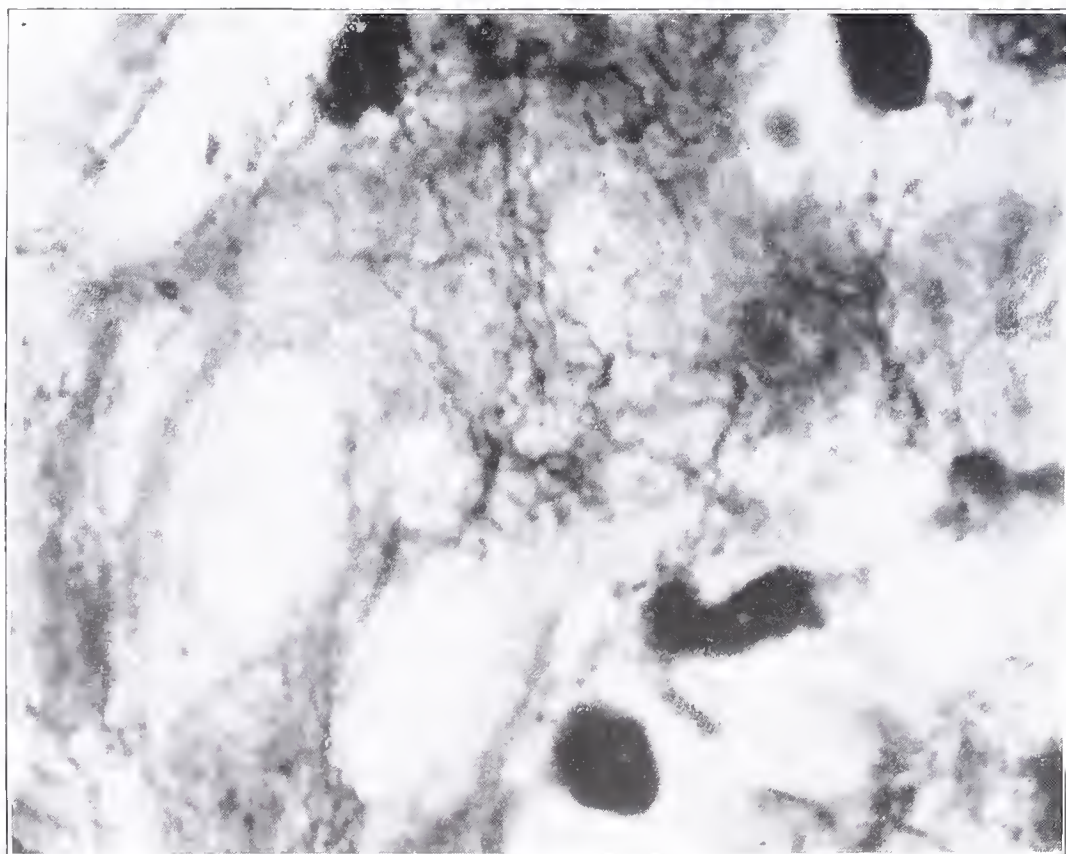


Fig. 20.—*Spirochaeta cuniculi* in tangled masses on surface of condylomatous lesion. Warthin-Starry silver-agar cover-glass method. B. & L. oil immersion 2 mm., compensatory ocular No. 4, bellows length 155 cm.

PLATE 11

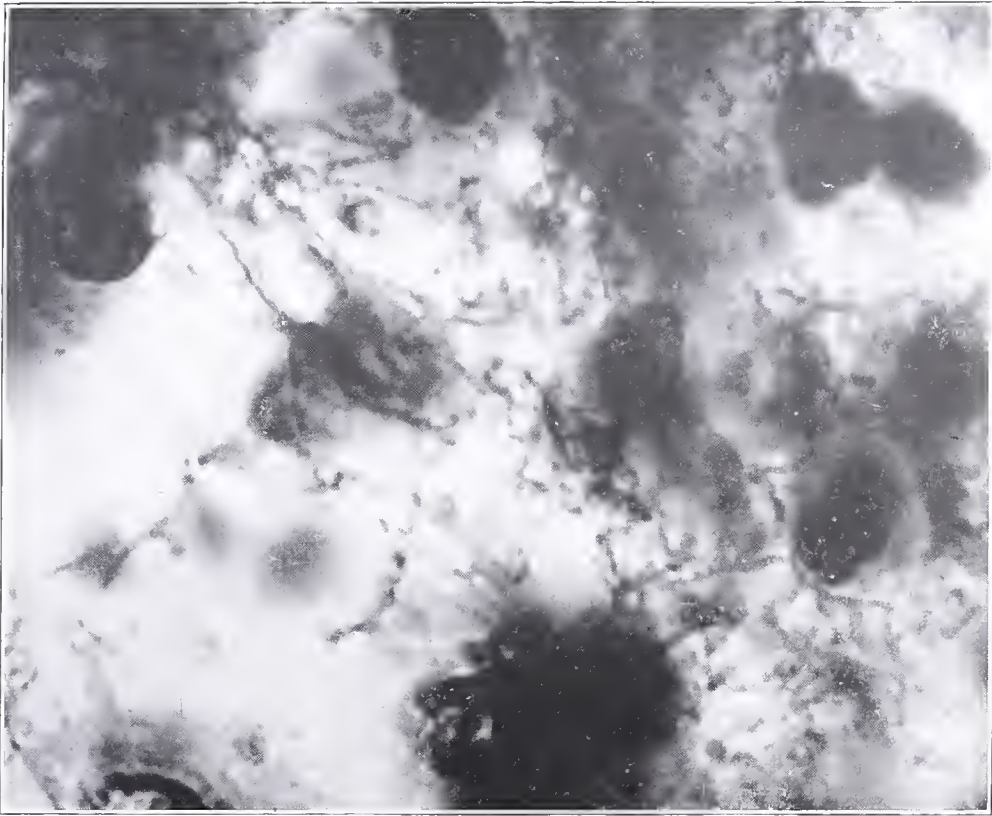


Fig. 21.—*Spirochaeta cuniculi* in tangled masses in the epidermis of condylomatous lesion. Same stain and magnification.



Fig. 22.—Single spirochete below epidermis. Same stain and magnification.

PLATE 12

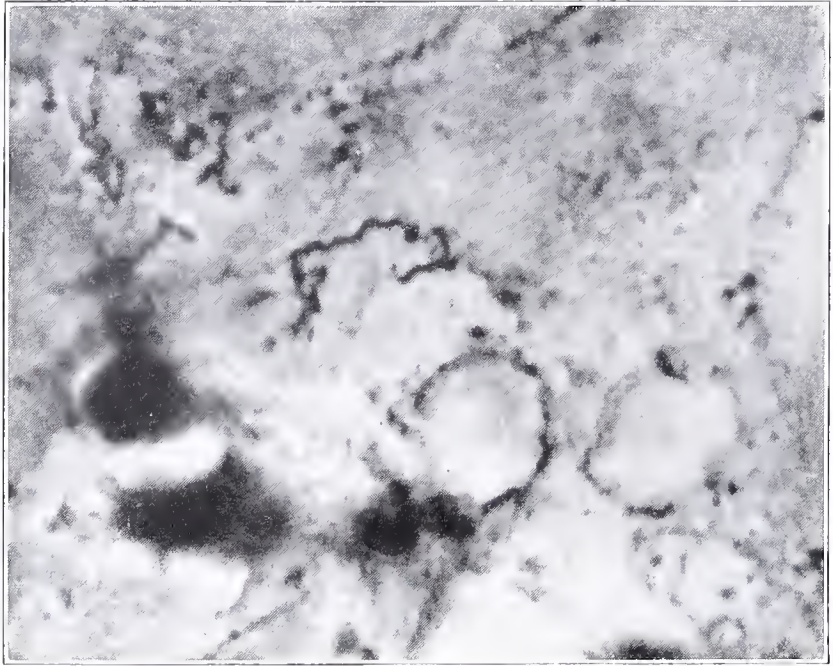


Fig. 23.—Numerous cuniculi organisms in upper portion of corium in condylomatous lesion. Same stain and magnification.

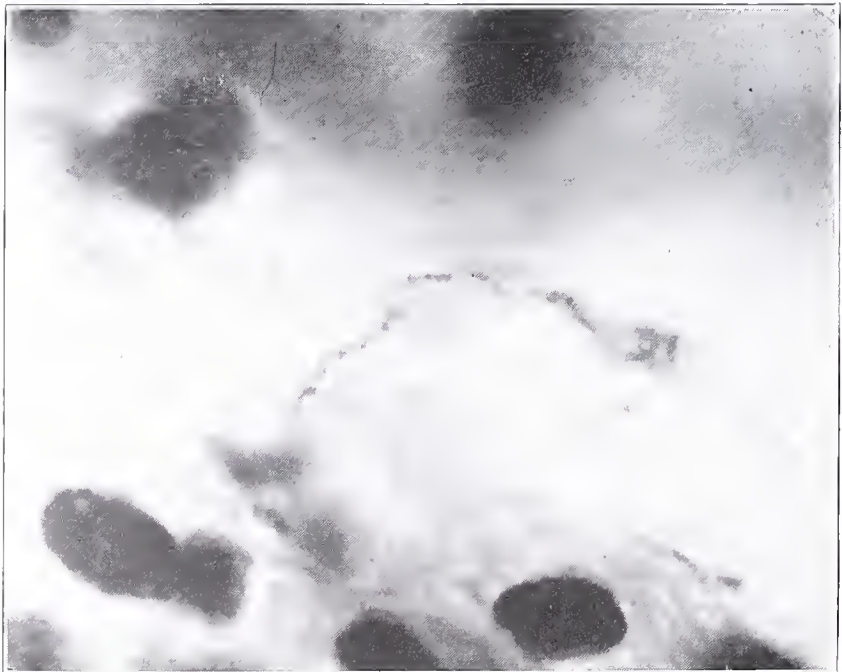


Fig. 24.—Group of cuniculi spirochetes in the submucosa of latent lesion. Same stain and magnification.

HYPERTROPHY OF THE HEMOLYMPH NODES IN TEXAS-FEVER IMMUNES

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At the Seventeenth International Congress of Medicine, in London, August, 1913, I made a preliminary report before the Section of General Pathology and Pathological Anatomy on "The Reaction of the Hemolymph Nodes to Chronic Protozoal Infection."¹ This report was concerned with a brief description of the changes occurring in the hemolymph nodes of man in chronic malaria, and in those of cattle in 2-year-old Texas-fever immunes. The present paper is intended to be a complete report on the latter condition, since, so far as I am concerned, this investigation has been carried as far as the resources of my laboratory will permit. In 1908, for the first time, my attention was called to the occurrence of a remarkable hypertrophy of the hemolymph nodes in Texas steers, by an old student, Dr. Clarence A. Good, of St. Joseph, Mo., who had observed it in the slaughter-houses of that city. A special study of the condition was immediately begun, but the supply of material from that source was soon closed, and it became impossible to complete the investigation at that time. Nevertheless, it has been carried on at intervals since, whenever opportunity has offered. The carcasses of steers have been examined for a similar occurrence of hemolymph node hypertrophy, both in local material and in that sent to the laboratory for this purpose by veterinarians living in various parts of the country. The total collected material represents chiefly the central, western and southwestern portions of the country. The descriptions of the pathology and conclusions given below are based on the examination of this material.

THE NORMAL HEMOLYMPH NODES OF CATTLE

There are normally 200 or 300 hemolymph or hemal nodes in cattle. They are found chiefly in the prevertebral fat as red or bluish red bodies contrasting sharply with the white fat. They vary in size from that of a pinpoint to that of a cherry or almond; the majority are about the size of small peas. In the regional lymph node areas, in the subcutaneous panniculus, and particularly about the joints, they are always found associated with ordinary lymph nodes, usually one to two or three

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¹ Proc. XVII Inter. Cong. of Med., 1913, Section III, Part II, p. 211.

red nodes about the size of a mustard seed lying in or near the hilus of the node, or even imbedded within the substance and surrounded by the capsule of the node. In the prevertebral thoracic region, the hemal nodes are often found imbedded within the tissue of the pale lymph node, so that transition forms seem to occur. In general, the hemolymph nodes in cattle are spherical, often with a slight hilus indentation on one side, into which vessels pass; sometimes they are bean or kidney shaped with a definite hilus; others may be almond shaped or elongated into cylindrical cords. The hemolymph nodes are frequently regarded by butchers as hemorrhages or blood clots. On microscopic examination, they are found to consist of blood sinuses and lymphoid tissue, with occasional connective tissue trabeculae. The sinuses vary much in size; they consist of a peripheral sinus, which is usually large and is traversed throughout by a loose network of reticulo-endothelial cells and fibers, through the large spaces of which the blood circulates. There are also usually central and communicating sinuses showing the same reticular network and large venous lacunae without such reticulum. The lymphoid tissue may be scanty or abundant; it is arranged in cords or trabeculae; there is usually no division into cortical and medullary portions, although some nodes may show this arrangement. Germ centers may be present in the lymphoid tissue, particularly in the neighborhood of the peripheral sinus in which they may often be observed. Large mononuclear phagocytes, hemophages in particular, and pigment-containing cells are found in the reticular spaces of the sinuses, especially in the central ones. Eosinophil and mast cells are usually present in numbers in the lymphoid tissue. Numerous relatively large sized vessels pierce the capsule and hilus. We are as ignorant of the function of these nodes as we are of that of the spleen. They seem to occupy a position intermediate between that of the spleen and of the bone-marrow. They are actively concerned in the phagocytosis of red blood cells and pigment transformation, also in the production of lymphocytes. The development of the reticulo-endothelial surface in the sinuses suggests a function of removing substances from the circulation and an active part in the protective activities of the body.

HYPERTROPHY OF THE HEMOLYMPH NODES OF THE STEER

In normal beef carcasses, no generalized hypertrophy of the hemolymph nodes has ever been found. Occasionally a single node, or two or three unusually large nodes have been seen without other accompanying pathologic conditions. Moderate hypertrophy has been noted in a number of cases of tuberculous perisplenitis with fibrosis of the spleen.

A marked general hypertrophy has been seen only in steers coming from the southwest. The great majority of these were 2-year-old Texas-fever immunes coming from the southern Pecos Valley region. The only exceptions were 2 collections of material from New Mexico and Oklahoma, 8 steers in all, said to be suffering from loco-disease. No positive history could be obtained as to the previous occurrence of Texas-fever in these animals, although it was admitted to be very probable. As these steers showed precisely the same pathologic conditions as those seen in the Texas-fever immunes, the probability becomes practically a certainty that they were all Texas-fever immunes, animals that had survived an attack of the infection, and had, thereby, gained an absolute immunity to further attacks. While the condition of marked hemolymph node hypertrophy occurred only in Texas-fever immunes, not all such animals showed it. It was found only in those animals showing a pathologic condition of the spleen.

MATERIAL

The material consisted of the prevertebral adipose tissue containing the hemolymph nodes removed in large strips or pieces, the regional lymph nodes, with neighboring adipose tissue, mesenteric and omental fat-tissue, spleen, and portions of other organs. The greater part of this material was fixed in 10% formol and shipped to the laboratory in large milk cans. From some of the cases material fixed also in Zenker's, mercuric chloride, alcohol, Müller's and Flemming's solutions, was obtained. Smears of the fresh glands were also studied. The usual laboratory technic was applied to the study of the material. Paraffin imbedding with staining of sections on cover-glasses was employed for the greater part of the tissue study. Ordinary stains were used for the tissue, Giemsa and others for the search for micro-organisms, and the various blood stains for special cell studies.

GROSS APPEARANCES

All hemolymph nodes in the affected animals showed a most striking hypertrophy. The regional hemolymph nodes, the cervical and subcutaneous, stood out in the fat tissues, enlarged 10-100 times their normal size, and instead of being found by searching, as is usually the case in normal animals, presented themselves as deep purplish red or black structures standing out in sharpest contrast to the fat and the light lymphatic nodes. Every regional node of the pale lymphatic type was found to have associated with it at least one, often several, hemolymph nodes, usually in its hilus, but not infrequently imbedded in the

substance of the lymph nodes itself. Such imbedded nodes usually presented a sharply circumscribed border or capsule, but in some cases there was no sharp transition between the hemolymphatic tissue and that of the lymph gland. The hilus hemolymph nodes of the regional lymphatic nodes that usually are not larger than a large pinhead or small mustard seed become in these cases as large as a large marrow-fat pea. Throughout the subcutaneous panniculus in which it is ordinarily difficult to find hemolymph nodes in normal steers, it was easy to find them in these cases, always, however, in close association with a pale lymphatic node. In the prevertebral fat, throughout its entire length, the solitary hemolymph nodes showed a still greater hypertrophy. The white fat appeared to have deep reddish brown or reddish blue-black spherical masses like large cherries or marbles scattered throughout and imbedded in it. Many were as large as walnuts, occasionally some were as large as an orange. They looked very much like small spleens or kidneys imbedded in the fat. The majority of them were spherical, many absolutely so, others with a slightly depressed hilus on one side, while others were decidedly bean or kidney shaped with a well marked hilus into which vessels passed. They all possessed a thin, delicate and tightly stretched capsule. In the fresh, unfixed state puncture or cutting of this capsule produced a free discharge of blood from the opening. On section, the enlarged nodes were found to consist chiefly of greatly dilated blood sinuses and venous lacunae, through which relatively small grayish trabeculae of lymphoid tissue ran. The peripheral sinus was always enlarged out of proportion to the other blood spaces. Compared to the size of the sinuses, the total amount of lymphoid tissue in the hypertrophic nodes was always very small.

The greatly increased size of the hemolymph nodes in these Texas steers is a great aid to the study of their anatomic relationships. If they are not increased in number, which increase seems doubtful, the total number of hemolymph nodes in the steer is much greater than has been estimated from the study of the normal animal. In the latter, the highest counts have been about 300, and in many animals the total number found is often much less, 75-100. In Texas-fever immunes, counts of 600-700 have been made in cases from which practically all of the adipose tissues were secured for examination. In the average case with incomplete material the counts averaged 200-300. A striking feature of the cases of hypertrophy is the fact that the hilus hemolymph nodes never exceed a large pea in size, while the solitary nodes of the prevertebral region become so much more strikingly enlarged. In the normal animal, however, it is often difficult to find the point-like

hilus nodes, while in the same case the prevertebral nodes are as large as peas. Without any mathematical accuracy, it is probable that the increase in size of both is relatively uniform. The pale lymph nodes of these animals are also hyperplastic, but not in proportion to the hemolymph node hypertrophy. They were also, in a number of the cases, much browner than the normal lymph nodes of the steer. With the magnification of the normal anatomic features, it is easy to settle a number of disputed points concerning the anatomy of the hemolymph nodes. One of these is the fact that every peripheral pale lymph node has associated with it one or more hilus red hemolymph nodes. This is true even of the rudimentary lymph nodes of the subcutaneous panniculus. In the case of the larger peripheral regional lymph nodes, the cervical nodes, and particularly those in the neighborhood of the joints, the hilus hemolymph nodes are larger and are usually more easily found in the normal animal, while in the cases of hypertrophy they were commonly of the size of large peas. In the prevertebral fat, the constant association of hemolymph node and lymphatic node does not hold as it does in the case of the peripheral nodes. In the thoracic region, many of the lymph nodes show large hilus nodes or imbedded hemolymphatic nodes; but in the retroperitoneal adipose tissues, the large hemolymphatic nodes are usually solitary, while close to the vertebrae the association of the two types is again evident. It has been denied by some writers that bean or kidney shaped hemolymphatic nodes occur, or that lymphatic glands ever present a mixture of lymphatic and hemolymphatic structures (A. W. Meyer). The cases of hypertrophy show numerous kidney-shaped hemolymph nodes, and also lymph nodes containing hemolymphatic areas. A new histologic point is, however, brought out here, in the fact that in the majority of these "mixed glands" the hemolymphatic area is distinctly encapsulated and appears within the pale lymph node as an "imbedded" or "included" structure.

MICROSCOPIC APPEARANCES

Sections of the hypertrophic nodes show microscopically a striking enlargement of all the structures of the nodes. All of the blood spaces, the central, communicating and peripheral sinuses, and the venous lacunae and blood vessels are greatly enlarged, particularly the peripheral sinus. Throughout these dilated sinuses extends a loose network of reticulo-endothelial fibers and cells, between which are large blood spaces packed with red cells. The appearances here favor the view that the sinus reticulum is an open one and not covered by a distinct layer of endothelial cells; on the other hand, the apparent fibers of the reticulum are connected with the cells themselves. The sinus reticulum, therefore, appears in these nodes as a fibrocellular network of anastomosing branching cells, a specialized reticulo-endothelium, through the meshes of which the blood circulates. In the meshes of this network relatively few hemophages

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were found. No blood pigment was seen in the reticulo-endothelial cells, and the iron reactions for hemosiderin were negative. Many of the cells in the reticulum appeared hypertrophic, and occasional mitotic division figures were seen. No developmental stages of piroplasma could be demonstrated in the reticular cells, although especial search was made for these by many staining methods. The lymphoid substance of the nodes presented also a marked hyperplasia. The germ centers were increased in number and size, and showed many mitotic figures, indicating increased activity in the production of lymphocytes. Little hemosiderin was found in the lymphoid tissue. No parasites could be demonstrated in it; and the staining of the smears made of the fresh hemolymph nodes was also negative. Nothing suggesting any stage of development of a protozoan organism, developmental stages, involution, or resting bodies could be demonstrated. The changes in the hemolymph nodes in these Texas-fever immunes represent, therefore, a uniform hypertrophy of lymphoid and sinus structures; in particular, a greatly increased reticulo-endothelial surface in the enlarged blood sinuses.

The ordinary lymph nodes from these cases were also hyperplastic, showing an increase in the number and size of the germ centers with many mitotic figures in the latter. The brownish nodes contained much hemosiderin, and great numbers of eosinophil cells, in these respects resembling the changes in the lymph nodes of goats and sheep after splenectomy. No parasites or parasite-like forms could be demonstrated in the lymph nodes or in smears made from their freshly cut surface. The microscopic examination of the spleens from these animals showed fibrosis, lymphoid atrophy, chronic passive congestion, and a greater degree of hemosiderosis. No parasites could be found in either smears or sections of the spleen. The examination of sections and smears of the bone-marrow was also negative as to parasites. The microscopic examination of other organs or tissues showed only chronic passive congestion and simple atrophy.

The above investigations carried out through a number of years established the following facts:

1. In many steers, Texas-fever immunes, coming from the southwest, but not in all, there occurs an extraordinary generalized hypertrophy of the hemolymph nodes.

2. Such a generalized hypertrophy of the hemolymph nodes has not been seen in any other American steer; it has been found only in animals known to have had Texas-fever, or in whom the possibility of such a previous infection could not be excluded.

These observations are apparently the first that have been made of this remarkable involvement of the hemolymph nodes in American Texas fever. I have been unable to find any reference to it in the papers on Texas fever written in this country; and in the textbook articles on this infection no mention is made of the hemolymph nodes in the statements given of its pathology.

The involvement of the hemolymph nodes in piroplasma infections has, however, been noted by K. F. Meyer in his studies on "East Coast

Fever" in South Africa.² Meyer found that following the bite of the tick, the agametes or metagametes localize in the hematopoietic organs, especially in the lymph nodes, hemolymph nodes, bone-marrow and spleen, where they are found in the developmental stage, and can be obtained by puncture of these tissues, and easily recognized as small roundish, intracellular, granular particles in the lymphocytes, mononuclear and endothelial cells. In these, they grow rapidly and form typical agamonts which are set free by the degeneration of the infected cells. Gamonts are then formed which break up into gametocytes by schizogony; these enter the red blood cells and appear in them as the parasite of East Coast fever (*Theileria parva*, Theiler; *Lymphohaematocytozoon parvum*, Meyer). Meyer states that the process of development is easily seen in preparations made from the hemolymph nodes of the infected steer: "The entering of the gametocytes into the red corpuscles is seen in every preparation made from the hemolymph nodes." Meyer found the hemolymph nodes hyperplastic in animals infected with East Coast fever, and was able to transmit the disease to susceptible animals by inoculations from such hyperplastic hemolymph nodes, even in cases in which no parasites could be demonstrated in the blood or lymphatic glands of the animal showing the hemolymph node hypertrophy. In a personal communication from Meyer in 1912, he described the hypertrophy of the hemolymph nodes in cattle infected with East Coast fever, as identical with that seen by us in American Texas-fever immunes.

It seems very probable, therefore, that the hemolymph nodes of cattle are involved to an unusual degree in piroplasmiasis, and that there is a special tendency of the part of the piroplasma, during its stages of development, and in its resting form most likely to be localized in the hemolymph nodes. Whether the continued hypertrophy of the nodes in Texas-fever immunes is due to a latent infection cannot be decided from our investigation. We were unable to carry out any inoculation experiments to settle this point, and this should be done by some laboratory accessible to fresh material. Although intensive search of our fixed material and of smears made from the fresh nodes failed to show anything resembling protozoan parasites, it must be remembered that Meyer was able to transmit East Coast fever by hemolymph node inoculation even when he could not demonstrate the presence of the parasite in the blood or the lymphatic nodes.

² Reports of the Veterinary-Bacteriologic Institute of the Transvaal, 1909, 1910; Proc Path. Soc., Phil., 1911, 14, p. 52.

It does not seem probable that the generalized hypertrophy of the hemolymph nodes in Texas-fever immunes can be due to a compensatory process for either the accompanying pathological changes in the spleen or for the increased destruction of red blood cells. In other affections of cattle with more marked fibrosis of spleen and with greater hemolysis than are shown in these animals that have survived Texas-fever, such a marked generalized hemolymph node hypertrophy does not occur. It seems reasonable to infer, therefore, that the hypertrophy in the Texas-fever immune is the manifestation of a protective reaction against some form of the parasite, resting stages probably, localized in these nodes. This question can be settled by inoculation experiments. The enlarged hemolymph nodes are not engaged in red blood cell regeneration, or in any unusual participation in blood-pigment transformation. Two definite facts are manifested in these large hemal nodes: The increased activity of lymphocyte production; and the greatly increased blood surface of the reticulo-endothelial system, undoubtedly a protective mechanism, both from the standpoint of phagocytosis and of antibody formation. It is reasonable to interpret these phenomena as the reaction to a latent infection and that these Texas-fever immunes with generalized hemolymph node hypertrophy are still parasite carriers. How long after the apparent recovery from the attack of Texas fever this hypertrophy and carrying of the organism persist, we cannot say positively. It has been found very difficult to obtain exact data on this point. The majority of the animals were said to have had the disease in early life. The one point of importance gained as the result of inquiries along this line is that the animals had sufficiently recovered from the attack of the infection, as to be more or less presentable ("fairly fat") for market. Since it does not occur in all animals who have survived an attack of Texas fever, it is probable the organisms disappear entirely within the bodies of such cases, and the hypertrophied nodes return to normal. No evidence of any other form of parasitic infection could be found in the animals showing the hemolymph-node hypertrophy.

SUMMARY

In many Texas-fever immunes there occurs a very marked generalized hypertrophy of the hemolymph nodes, which is to be interpreted as a manifestation of protective reactions against a latent infection. Inasmuch as Meyer has emphasized the involvement of the hemolymph nodes in East Coast fever, there appears to be a special predilection for these organs in piroplasma infections.

PLATE 1



Fig. 1.—Hypertrophic haemolymph nodes from prevertebral fat of Texas-fever immune. Natural size.

PLATE 2



Fig. 2.—Hypertrophic haemolymph nodes from prevertebral fat of Texas-fever immune. The lower node on the 1 shows a characteristic kidney shape with well defined hilus. Natural size.

PLATE 3

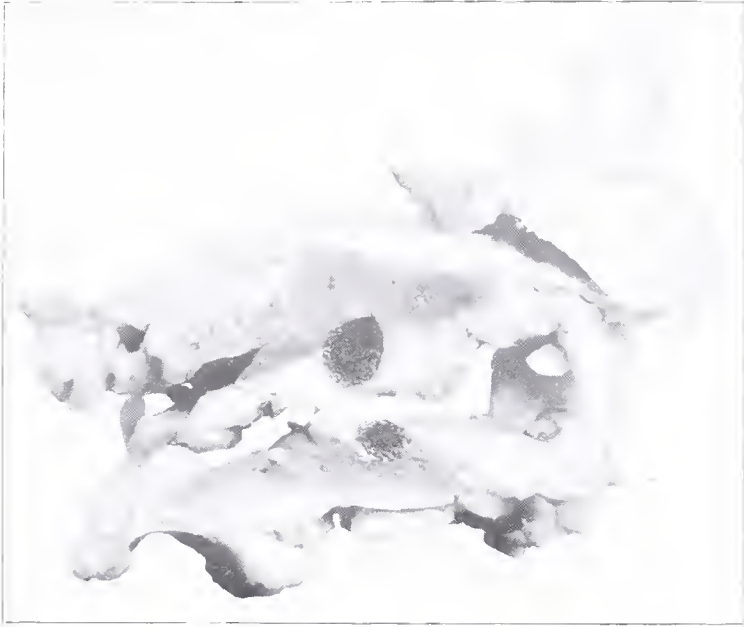


Fig. 3.—Hypertrophic haemolymph node in hilus of peripheral lymph node, from Texas-fever immune. Natural size.

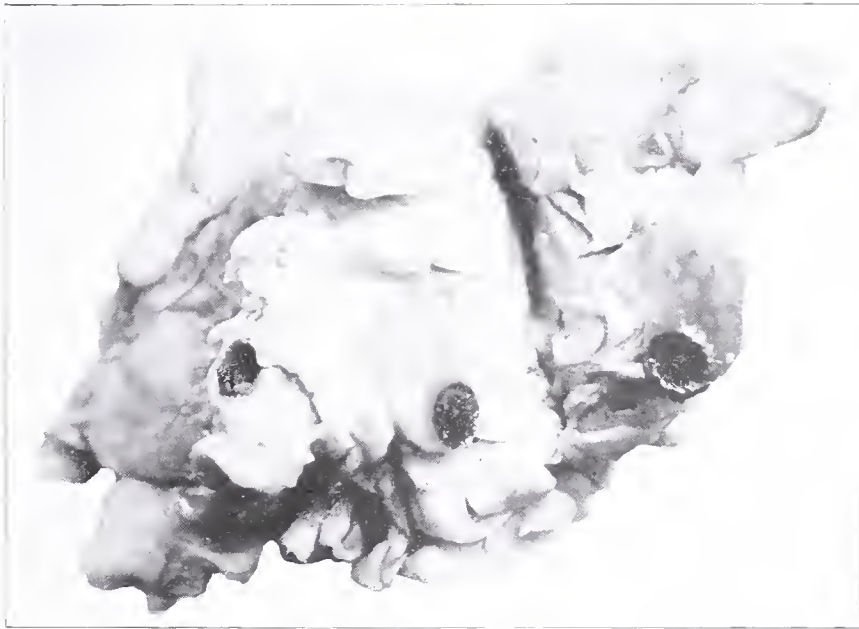


Fig. 4.—Cross-section of peripheral lymph node showing cross-section of haemolymph node imbedded within the capsule of the former. From Texas-fever immune. Natural size.

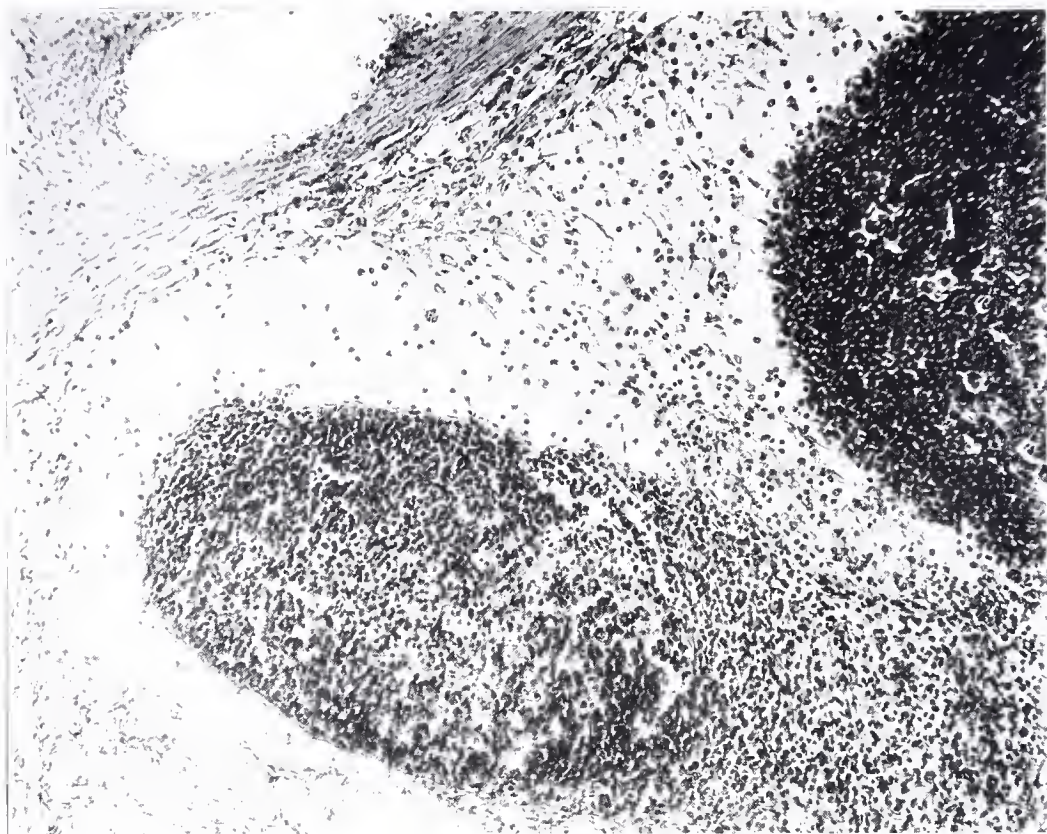


Fig. 5.—Section showing portion of peripheral sinus of hypertrophic haemolymph node from two-year-old Texas-fever immune. Hyperplasia of reticulo-endothelium of enlarged sinus; and hyperplastic germinal center extending out into sinus. Spaces between reticulo-endothelial cells and fibers are packed with red blood cells.

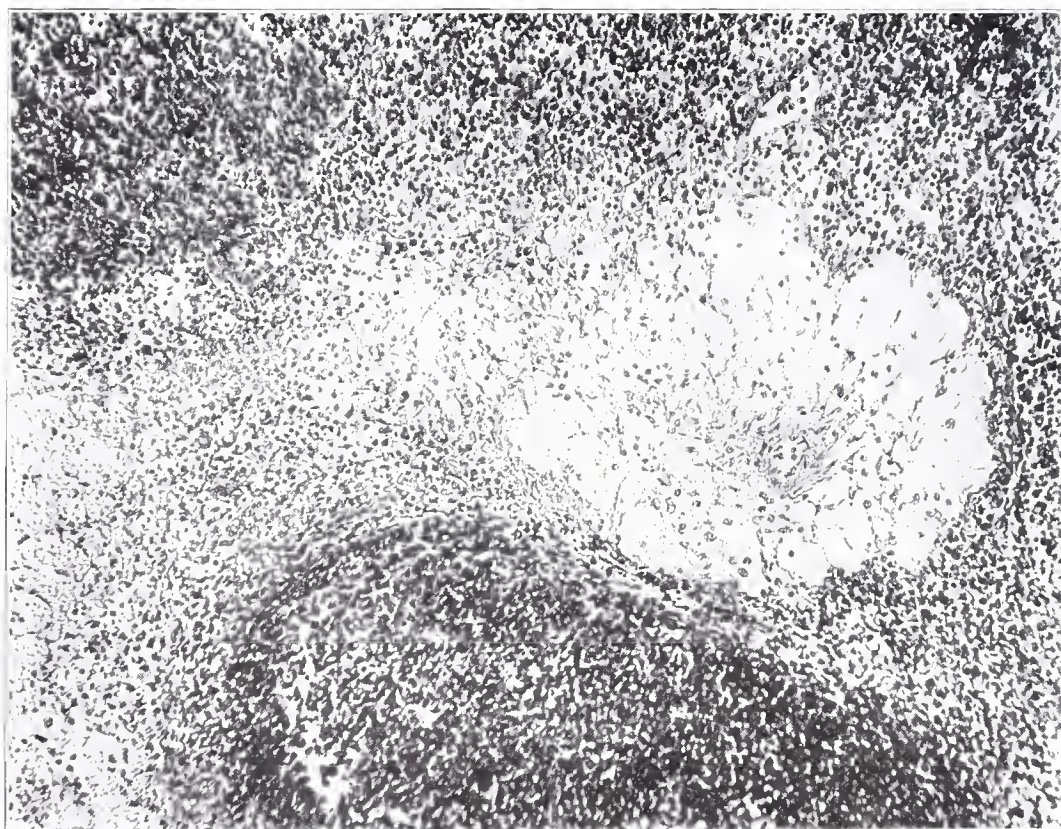


Fig. 6.—One of the communicating sinuses of same node, showing same distention of the blood spaces of the reticulo-endothelial network.

BACTERIAL PARASITISM, BACTERIAL PATHOGENISM, AND RESISTANCE TO BACTERIAL INFECTION

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The history of bacteriology goes back less than 50 years, yet it is conservative to claim that the developments of this scant half century have been of greater import to mankind than those of any preceding period. It is obvious that man, even from the earliest days, has been more than the peer of the microbe; otherwise, the human race would have perished from the earth long ago. With the advance of bacteriology and immunology during the last few decades, however, man is not only gaining mastery over his hidden foes, he is even harnessing microbic activity to labor in his interest.

Among the many lines of attack which have advanced the frontiers of knowledge of bacterial activity, none has been found more opportune than that one which leads to the unfolding of those complex, reciprocal relations between host and microbe which comprise the domain of immunology. The significant events which have contributed most definitely to this progress center around the labors of two great scientists, Metchnikoff and Ehrlich, the one a biologist, the other a biochemist. Out of their fundamental studies, two theories have been evolved which offer unlike explanations for the phenomena of immunity. Experiments and counter-experiments of ever-increasing refinement have led to some modifications of each theory; these, however, are of detail rather than principle; and at the present time the rival theories appear to be each more firmly entrenched than ever before, without any significant indications of a basis for reconciliation. It seems quite improbable that the vast amount of experimental work supporting these two theories should be found to be sufficiently inexact to discredit one or the other. Indeed, available evidence suggests that the reverse is the case. The experience of the past in other branches of science points to another possibility, namely, that the Metchnikoff and Ehrlich theories after all may not be rival postulates, but interpretations of two sets of phenomena.

Two significant facts stand out clearly if the controversy is studied from this angle. In the first place, the problem of immunity has been

approached by both factions from the point of view of the host. This is only natural, since the interest centers in the host rather than in the microbe. Also, the results of infection have been considered without much regard for certain essential phenomena in the cycles of perpetuation of the infecting agents, thus overlooking or disregarding the starting point of the process.

It is necessary to refer at this point to the salient features of the respective explanations of immunity.¹ The Ehrlich or humoral theory ascribes the phenomena of immunity to the persistence of soluble specific substances in the blood stream which are reminiscent of a successful struggle of the host against a specific invading microbe. These specific, soluble substances—specific antibodies—do not exist ordinarily in measurable amounts in normal, uninvaded persons; they develop during the progress of the conflict. These antibodies, therefore, are specifically designed munitions which eventually exterminate the foe within the frontiers of the body. Their persistence in the blood stream protects the body against subsequent invasion by the same organism.

It is important to recall that the bacteria which leave these imprints of invasion are those which belong for the most part to that group formerly designated the "contagious bacteria." They incite clinically specific disease. They develop within the tissues of the body as an essential part of their infective cycle, and they possess the ability of passing from host to host unaided. Also, they enter the tissues at specific, well-defined points. Phagocytic elements play no permanent part in the phenomena of immunity to contagious bacteria, so far as available information indicates.

The other theory of immunity, the Metchnikoff or cellular theory, emphasizes the part played by the leukocytes, and, to a lesser extent, by certain fixed phagocytic cells of the parenchymatous organs, in the defense of the body against infection. According to this theory, the invading microbes are attacked, engulfed and destroyed by cellular elements found chiefly in the blood and lymph channels. The body mobilizes its leukocyte army during the invasion, and when the struggle is over, the excess cellular elements disappear. The same cellular elements—leukocytes—struggle against the most diverse microbic invaders. The leukocytes are short-lived; they cannot, as veterans, be regarded as a trained nucleus for another defensive cellular army. There seems to be no demonstrable quantitative or qualitative difference in the body after recovery from invasion to mark the victory which

¹ Acquired, not natural or inherited, immunity is of course referred to.

has been won.² Recovery from invasion does not seem to leave specific immunity against a subsequent invasion by the same microbe—indeed, there is rather a tendency toward increased susceptibility after recovery from infection.

Three additional factors deserve mention at this point. First, the bacteria which seem to be particularly assailed by the cellular defense of the body do not as a rule belong to the group of microbes ordinarily included in the group of "contagious" or infectious bacteria. They are more commonly normal parasitic bacteria living on the exposed surfaces or mucous membranes of the host. Second, they may enter the tissues at any point where a preexisting breach in the continuity of the limiting integument or mucous surfaces offer a portal of entry to underlying tissues. Indication of infection occurs, or may occur, almost immediately after the parasitic bacteria gain entrance to the tissues. A latent period following invasion of the tissues is not a significant event. Finally, those microbes which are susceptible to phagocytic activities of leukocytes do not ordinarily produce specific clinical syndromes, nor do they cause pathologic changes which are individually distinctive, as is the case in typhoid, for example. Rather, the lesions associated with their growth in the tissues belong to the group of general inflammatory reactions. Furthermore, the healing process is almost always associated with the formation of scar tissue.

If the broad facts of the cellular and the humoral theories are contemplated apart from technical details, it is possible to reconcile their operations with the reaction of the host against two rather distinct types of microbic infection, namely, a defense of the body against invasion by bacteria that live normally as parasites on the surfaces and mucous membranes—the parasitic bacteria—and a specific offense of the body against those microbes which cause progressive disease from host to host—the progressively pathogenic microbes.³ A brief consideration of the salient features of bacterial parasitism and of bacterial pathogenism will indicate the grounds on which the apparently rival theories are reconcilable with these two types of bacterial infection.

² The persistence of specific opsonins which resist an exposure to 60 C. for half an hour, following the repeated injection of alien cells—bacteria, blood cells, and others—is not necessarily a phenomenon involving the cellular theory of immunity. In fact, the evidence points to the association of these soluble specific substances with the humoral theory of immunity. Specific opsonins have not been demonstrated satisfactorily in naturally occurring infections by most parasitic bacteria.

³ The terms "parasitic" and "pathogenic" applied to bacteria are indiscriminately used; doubtless more accurate appellations will be coined when the facts of parasitism and pathogenism are better understood. For purposes of discussion the definitions which follow will be sufficiently exact to indicate the principle underlying their usage in this communication.

The host seemingly must be prepared to cope with two principal kinds of infection; first, by bacteria that are normally parasitic on the surfaces of the body and only accidentally invade the tissues, and, second, those bacteria that are exogenous and therefore alien to the body, but which appear to require the pabulum afforded by certain tissues for their nutrition and multiplication. The former are surface growers, and "opportunists" with reference to infection; the latter are tissue invaders.⁴ The life cycles of these two general classes of humanized bacteria are quite different. Each will be discussed separately to elucidate these differences.

THE LIFE CYCLE OF PARASITIC BACTERIA⁵

The essential and significant life cycle of parasitic bacteria which live normally on the skin and mucous membranes of the sound body consists of three distinct steps: First, multiplication on the host or in some channel in free communication with the surface of the body; second, escape from the body; and, finally, arrival on other suitable hosts. Many of these parasitic bacteria, as streptococci and staphylococci, are endowed with the ability of growing in the tissues of their host, but they lack the two essential potentialities which permit of their cycle being continued within the tissues of the body: first, an inability of themselves to force an entrance through the intact integuments which normally constrain them to live as surface parasites; and, second, even if they do penetrate the tissues through breaks in the integuments or by intracurrent disease, they lack the inherent ability of escaping from the tissues to the outside. It is chiefly because of these limitations that epidemics of furuncles, for example, do not occur from man to man. In other words, the temporary assumption of growth in the tissues is fatal to the perpetuation of the parasitic microbe. They are opportunists with reference to infection. There is no channel for the escape of interned parasitic bacteria to the outside world. It should be remarked in passing that staphylococci do settle down occasionally on the heart valves or in periosteal tissue, and streptococci do sometimes become chronic infectants of joints or other internal tissues. Epidemic heart or joint infections are not to be dreaded.

⁴ Tetanus, anthrax, botulism, and other microbes that could live if mankind were exterminated do not enter directly into this discussion. The first two just mentioned, however, are opportunists with reference to human infection. The latter overwhelms the body with its toxin. It is apparently no more contagious than mercuric chlorid.

⁵ Theobald Smith: *Amer. Med.*, 1904, 8, p. 711. Kendall: *Boston Med. & Surg. Jour.*, 1913, 169, p. 479; *Bacteriology, General, Pathological, and Intestinal*, 2nd Ed., 1921, Chapter 5.

The end is extinction of the microbe irrespective of the fate of the host.

The parasitic bacteria, because of their widespread distribution along the frontiers of the tissues of the body, are a constant menace to underlying tissues. They may enter at any point. The tip of the nose or the end of the toe is equally vulnerable. No appreciable latent or incubation period intervenes characteristically between invasion and the manifestations of infection. The body has, presumably by pre-ancestral evolution, perfected a defense for the tissues against this threat of invasion. The highways and byways of the body—the lymph and blood channels—are patrolled constantly by a mobile force of leukocytes—wandering cells which can arrest, engulf and digest moderate numbers of these normal resident parasitic bacteria which accidentally become invasive. The activities of these phagocytic cells are probably reinforced by the limited, nonspecific bactericidal properties of the body cells and fluids.

It is significant that the principal protection of the body against these opportunists is a nonspecific defense. The leukocytes and other phagocytic cells which gather in ever-increasing numbers in support of the invaded tissues are, so far as known, the same kind of cells irrespective of the parasite, be it staphylococcus or streptococcus.⁶ If the struggle ends with the overthrow of the invader, the cellular constabulary produced in excess of the normal needs melts away, and the body then seems to be qualitatively and quantitatively about the same as if no invasion had occurred.

The absence of well-defined immunity following recovery from microbic invasion which is quelled by the cellular elements, and the failure of many investigators to demonstrate any specific residuum antagonistic to the invaders, are supplementary evidences of the plausibility of this explanation of the function of leukocytes in the defense of the body.

The Metchnikoff or cellular theory explains these salient details of the defense of the body against parasitic invasion with considerable definiteness. Speaking in general terms, it would appear therefore that the cellular or Metchnikoff theory is not a theory of immunity [a theory of offense] but in reality a theory of defense.

⁶ Rapidly fatal streptococcus septicemia might seem to be an exception to this statement. In reality, the difference between the most virulent streptococcus infection and the most localized is quantitative in so far as the body is concerned, rather than qualitative. All gradations are readily recognized.

THE LIFE CYCLE OF PATHOGENIC BACTERIA

In sharp contrast to the simple cycle of perpetuation of the parasitic bacteria stands the relatively complex cycle of perpetuation of the progressively pathogenic bacteria. The pathogenic bacteria usually require a definite animal species for their host, but they are not ordinarily permanent residents on that host. Nevertheless, the essential part of their history—that of multiplication—is within the tissues of their host. They must complete serially a complex cycle to perpetuate themselves. The essential steps are: to reach a suitable host; to locate a specific portal of entry to definite underlying tissues of that host; to overthrow the defenses of the host at that point, which includes passage through intact tissue, and entrance to the tissues; growth in the tissues; and finally escape from the tissues to some channel communicating with the outside in sufficient numbers to reach other susceptible hosts before the initial host succumbs, or casts out the tissue-growing organisms. Each of these steps must be completed in the proper sequence to perpetuate the strain.

Many interesting fields for speculation, including balanced pathogenesis between host and microbe,⁷ serologic strains, and other natural phenomena present themselves at this point, but they are not germane to the main facts and therefore they are passed over.

From the point of view of the host, invasion by progressively pathogenic bacteria presents problems that a simple defense, as that of the leukocytes, cannot circumvent. The invader does not appear at any point—some one tissue is the point of attack. In naturally occurring infections this tissue, which restrains the passage of parasitic microbes, succumbs to the onslaught of the microbe in question; that is to say, the integument and natural defenses of the average susceptible host are inadequate to keep the prospective invader out. All the defenses fail and fall. The microbe then enters the tissues apparently unopposed and grows there, for a period of time at least, without apparent restraint. This is the incubation period. At the end of this period of time, frequently 10 days or even 2 weeks, a new phase in the struggle becomes noticeable, and specific substances appear in the blood stream of the invaded host which are specially designed offensive weapons forged to cope with the particular microbe. These accumulate in ever-increasing amounts and in favorable instances completely overthrow the alien microbe. Meanwhile, however, the descendants of the microbe

⁷ Kendall: Footnote 5.

have escaped from the tissues in large numbers and are on their way to new hosts. The antibodies which persist in the blood stream represent an unused excess of these specific munitions. They are a guarantee that a fresh invasion by the specific microbe shall be impossible, or nearly so.

The Ehrlich theory—the humoral theory—so it would seem, explains this immunity to reinfection through specific antibody formation with considerable exactitude, and therefore it is a true theory of immunity. It furnishes little or no clue to the normal defenses of the body.

One of the most puzzling and inexplicable problems arising from this discussion is an adequate explanation for the vulnerability of certain specific tissues to the attack of pathogenic bacteria. The intestinal mucosa, for example, resists the passage of the multitude of parasitic intestinal microbes year after year; yet it seems to offer comparatively little resistance at Peyer's patches to the entrance of typhoid bacilli. Of course the defense against invasion at this point includes not only the intact integument, it is reinforced by mobile and fixed phagocytic elements and by the nonspecific bactericidal properties of the blood as well. Neither the cellular nor the humoral theory, nor the so-called aggressin theory, explains the phenomenon satisfactorily. This is one of the many unsolved problems of infection which await elucidation.

Before entering on a discussion of certain kinds of infection which do not at first sight fit into this attempt to reconcile the cellular and humoral theories in their relations to microbic infection, the salient features so far elucidated are tabulated in brief for purposes of orientation:

PARASITIC BACTERIA

1. Normal residents of the body. They multiply ordinarily on the surface of the body, or in channels of the body communicating freely with the outside.
2. Cycle of parasitism: The parasitic microbe must (*a*) reach the surface of a suitable host; (*b*) multiply on the surface of the host; (*c*) escape in increased numbers to other suitable hosts.

PROGRESSIVELY PATHOGENIC BACTERIA

1. Exogenous microbes. They are not found on the normal body (except possibly in carriers), but multiply nevertheless within specific tissues of the body.
2. Cycle of pathogenism: The pathogenic microbe must (*a*) reach a suitable host; (*b*) penetrate to a suitable portal of entry; (*c*) force an entrance through this portal to the underlying tissues; (*d*) grow in the tissues; (*e*) escape from the tissues in greatly increased numbers to some channel opening to the outside world; and thence (*f*) reach other suitable hosts.

3. The normal intact integument of the body obstructs the entrance of parasitic bacteria to underlying tissues.
4. Invasion may occur at any point.
5. Excursion into the tissues is usually fatal to the perpetuation of the invading strain.
6. The normal, defensive mechanism of the body—free and fixed phagocytic cells—meets the invader at every point of entrance.
7. The phenomena of infection follow immediately, or very soon after, invasion by parasitic bacteria.
8. Recovery from infection usually leaves no discernible traces of the infection.
9. Scar tissue formation is usually a factor in the healing process.
10. Usually there is no lasting immunity following recovery—there may even be increased susceptibility to reinfection.
3. Specific parts of the integument are vulnerable to pathogenic bacteria. These are chiefly mucous membranes.
4. Invasion occurs at a specific point.
5. Invasion of the tissues is apparently essential to the perpetuation of the strain.
6. The normal defensive mechanism of the body—free and fixed phagocytic cells—falls before the assault of the microbe at the specific portal of entry.
7. A latent period of a week or more usually intervenes between the entrance of the pathogen into the tissues and the onset of the symptoms of disease.
8. Recovery from infection usually is associated with the persistence of specific antibodies.
9. The tissues usually return to normal with recovery.
10. Immunity is usually pronounced and enduring.

Prominent among the infections which do not at first sight seem to fit into this reclassification is the group of the pneumonias. There appears to be no well-defined pathologic or bacteriologic or immunologic basis for classifying pneumonias elsewhere than in the group of diseases incited by parasitic microbes, however. The pathology of pneumonia is essentially that of an inflammation. The tissues of the lung are distended by congestion, but the principal growth of the microbes is in the serum-filled alveolar spaces. Those that enter the blood stream presumably perish there. The incubation period is, or may be, very brief. Thus, pneumonia following exposure and postoperative pneumonia, neither of which differ essentially from lobar pneumonia, except possibly in the serologic type of the microbe,⁸ have a very brief incubation period. The leukocyte defense of the body, the absence of persistent antibodies following recovery, and the apparent increased susceptibility of the patient to reinfection, all suggest the parasitic relationship rather than the true progressive pathogenic disease of the

⁸ The serologic types of Cole are referred to.

Ehrlich type. The crisis in typical pneumonia is not explainable by any known facts.

Immunity to infections of the respiratory tract in general offers a field for future study. Infections of the respiratory tract are most contagious—witness the common cold—and most difficult to control. In general, bacteria that are found on the respiratory mucosa (including the mouth and nose) are so readily distributed from host to host by droplet infection that the effects of cold, and other environmental factors that influence the permeability of the respiratory mucosa must play a not inconsiderable part in opening the pathway for invasion with comparatively little specialization toward invasiveness by the microbes. As many of the infections of the respiratory tract are in the respiratory channels rather than within the actual tissues, the escape of these microbes is greatly facilitated.

The direct contact afforded by infected droplets from patient to victim contrasts strikingly with the circuitous path of the typhoid bacillus from one typhoid patient to another through an infected water supply, for example. Microbes fresh from the alveoli may reach prospective victims in considerable numbers through infected droplets, and intra-current environmental conditions may well afford that slight additional factor necessary or sufficient to start the new infection when such microbes are expelled by coughing and pass to the alveoli of the next in line. All of the epidemiologic criteria may thus be fulfilled, even though the microbes multiply in serum filled alveoli, or higher up in the tract, without actual invasion of the tissues necessarily taking place. In this manner temporarily widespread affections may arise, due presumably to a compounding of environmental conditions and opportunism on the part of the microbe.

A somewhat intermediate condition occurs with the meningococcus. Immunity to meningococcus reinfection is not clearly demonstrated. Serum therapy has been quite successful, especially when regard for the serologic strains of the organism is taken into account in selecting a serum. No inconsiderable factor in this success seems to be attributable to the accessibility of the inflamed area to direct medication. The organism seems to possess but slight invasive powers, and the carriers far outnumber the cases, even in epidemic periods. Colds and respiratory disturbances of similar nature seem to increase the percentage of cases, presumably by increasing somewhat the permeability of the respiratory mucosa.

On the other hand, influenza, whooping cough and possibly the exanthems, which are also supposed to enter the body through the respiratory tract, usually leave a well-defined immunity following recovery. One of the possible immediate effects of infection with the unknown exanthematous viruses is the opportunity afforded for entrance into the actual tissues of the body of resident streptococci and other parasitic microbes through a weakening of the respiratory barriers by the action of the primary infectant. These resident parasites in turn are responsible for much of the severity of the symptoms. Immunity, according to this supposition, would be a dual effect in that the body is protected by the unknown but specific antibodies which prevent the re-entrance of the primary virus (exanthems), and this immunity in turn prevents the lowering of the resistance of the respiratory mucosa to a point where the resident parasitic flora can gain entrance.

So-called epidemic sore throat, or milk-borne streptococcus sore throat, might seem to be a borderline infection, not readily allocated to either the parasitic or the true pathogenic types of infection. As secondary cases do not commonly arise from primary cases, however, it would seem that there is little evidence that a true tissue invading strain of streptococcus is created.

Diphtheria is another infective agent that presents some difficulty in classification. The production of antitoxin by the body in response to the absorbed toxin points clearly to the importance of a specific soluble antibody which eventually rids the tissues of the poison. On the other hand, the neutralization of the toxin does not necessarily destroy the microbe. Tetanus toxin similarly stimulates specific antitoxin formation, although the tetanus bacillus is not necessarily even a humanized parasite. Finally, the toxin of *Bacillus botulinus* overwhelms the body even though the poison is formed wholly outside the body. There is no indication that *Bacillus botulinus* is a human pathogen; indeed, it grows rather better at 30 C. than at 37 C., the body temperature. The question arises: Should diphtheria bacilli be regarded as parasitic microbes—surface growers—forming a soluble toxin which may become a formidable menace to the body? The non-toxin-producing strains furnish a connecting link from this point of view between relatively innocuous strains (which may again become toxicogenic) and toxin-producing strains which frequently cause serious illness, even though the microbe is not habitually a true tissue invading

strain. Another possibility presents itself: Is soluble toxin formation necessarily a criterion for admission to the group of progressively pathogenic bacteria? Tetanus and botulinus suggest the reverse is the case.

This focuses the attention sharply on a difference between surface growing and tissue invading microbes, on the one hand, and the soluble toxin producing bacteria, on the other. In the case of the accidental and habitual invaders, the microbe is the focus of conflict and the success of the host is measured very largely by the elimination of the microbe. The reaction of the body against tetanus, diphtheria, botulinus toxins, as well as ricin and abrin, is the formation of a specific chemical antidote.

Tuberculosis and leprosy are infections that do not seem to fit into current ideas of either the humoral or the cellular theories. The invasiveness of the tubercle bacillus group, together with the avascularity of the typical lesions, and the characteristically long drawn out battle between host and microbe before the former falls, is indicative in part at least of a new angle to the discussion which does not receive adequate rejoinder from either theory. The cells of the body which form the tubercle seem to be a not unimportant factor. Immunity does not seem to play much of a part. Further knowledge is needed before the question can be answered satisfactorily.

The discussion thus far includes bacteria which perpetuate themselves chiefly through their adaptation to conditions on or in specific hosts which they reach and escape from without directive participation in the transmission by the host itself.

Contact infections, of which gonorrhea and syphilis are familiar examples, are departures from the types already discussed in that the infected host implants the microbe directly into vulnerable tissues of succeeding hosts. Both the gonococcus⁹ and *Treponema pallidum* appear to enter the tissues through minute abrasions. Inoculation is quite as direct in the transmission of venereal disease from host to host as is inoculation in the laboratory for the perpetuation of a laboratory culture. In this manner is the specific venereal flora perpetuated. Otherwise the microbes would in all probability perish.

There seems to be no clearly discernible immunity to gonorrhea, and the cellular defense of the body plays the most conspicuous part in the defense of the host against the extension of the infection. The immunity of syphilis is not thoroughly worked out. The primary and

⁹ The conjunctiva may prove to be an exception.

secondary lesions, but not ordinarily the tertiary lesion, are significant in the perpetuation of the treponemes.

Bubonic plague is illustrative of a somewhat similar type of infection in that the microbe, *Bacillus pestis*, does not ordinarily pass unaided from host to host;¹⁰ it requires the agency of the flea. The growth of the bacilli in the blood stream of the infected host furnishes the requisite condition for insect transmission, which may take place from rodent to rodent, or from rodent to man.

Bacterial vaccines deserve a word of comment. They are introduced into the tissues for two principal purposes; to increase specific resistance to infection, and to assist invaded tissues to overcome infection. The typhoid-paratyphoid vaccines are preeminently the best known and most thoroughly studied examples of the first kind. It is only necessary to recall the typhoid incidence in the American army during the Spanish American War and then that of the troops of all the nations during the World War to be convinced of their efficacy. Typhoid-paratyphoid vaccines are of doubtful or unproved value as curative agents.

In the use of these vaccines for purposes of immunization, it is noteworthy that a latent period of several days elapses between the last injection and the maturation of the specific antibodies. This is reminiscent of the incubation period of typhoid fever. There seems to be a reduction in the normal resistance of the host to infection during the earlier days of this latent period immediately after the injection of the vaccine and before the appearance of the specific protective antibodies.

In contrast to this protective vaccination with typhoid bacilli stands the curative value of staphylococcus vaccines. Protective vaccination against furuncles is of unknown or dubious value, but frequently successful results are obtained from the injection of staphylococcus vaccines in the treatment of furunculosis. Here the effects are obtained rather quickly in favorable cases. The latent period is rather brief, and the beneficial effects seem to appear rather quickly in many instances. These results suggest a possible relationship with nonspecific protein therapy.

Typhoid prophylactic vaccination and staphylococcus vaccination provoke diametrically opposite reactions. Various speculations can be

¹⁰ Pneumonic plague is of course directly transmissible from host to host by droplet infection. So far as is known, pneumonic plague is limited to human transmission.

indulged in to account for these apparent differences, but unfortunately they fail to furnish an adequate explanation. One factor, however, deserves mention because it emphasizes a difference between parasitic and progressively pathogenic bacteria from an angle not previously considered. It is believed that cholera and typhoid microbes, injected into subcutaneous tissues, tend to find their way to the alimentary canal, the organ in which they operate in their initial skirmish with the body. There is no evidence, on the contrary, suggesting that staphylococci similarly introduced into subcutaneous tissues tend to migrate to any special tissue or organ. If these claims be substantiated, there is abundant opportunity for theorizing on the part played by chemotaxis in determining the path followed by certain kinds of bacteria into special tissues. It cannot be advanced as an attractive force acting without the tissues, however; it would be audacious to claim that typhoid bacilli, for example, would respond to the chemotactic urge of the Peyer's patches when they had merely lodged in the mouth.

Nevertheless, tetanus toxin passes to the central nervous system from almost any tissue of the body, as does the botulism toxin. Diphtheria toxin, on the contrary, exerts its baneful effect largely on parenchymatous organs, although the post-diphtheritic paralyses seem to involve the neurologic machinery. Leukocytes also seem to be responsive to chemotactic stimuli; they may be attracted or repelled.

To return to staphylococcus vaccines, it seems quite probable, although experimental evidence is not clear on this point, that injections of killed staphylococci, like those of typhoid bacilli, should stimulate antibody formation. If this should prove to be the case, it would appear that there may be after all an artificial means of enlisting the offense of the humoral type in aid of the natural defensive forces of the body. It is proceeding too far into speculative relations, however, to prolong this discussion of unexplored fields, when the primary intent is not to add new facts or theories to existing information. All that is contemplated is to introduce a new and possibly more comprehensive point of view into current discussions.

This point of view contemplates microbic infection and macrobic resistance to infection as a duel between a micro-organism and a macro-organism, in which the former is the aggressor, at the start at least. The menace of the microbe is convergent toward two somewhat distinct and definite types, namely, that of the opportunist, or parasite, which lives normally an inconspicuous existence on the surface of the body

of the host, or in channels or cavities opening to the surface, and that of the exogenous, or pathogenic organism, which is a finished criminal.

Excursion into the tissues of the host is ordinarily fatal to the perpetuation of the opportunist strain. Growth in special tissues is seemingly an essential characteristic of the progressively pathogenic microbe. Many apparent exceptions to this proximate classification may be thought of which require mental dexterity to fit into one or the other of these principal types of invaders. In the light of present incomplete knowledge of the underlying causes for the manifold manifestations of infection and the sequelae thereof, it is safe to leave the explanation of these incompatible cases to experimentation rather than speculation.

It is not probable that the point of view herewith presented is to be regarded as other than an hypothesis having considerable evidence, experimental and otherwise, in its favor. This is equally true for nearly all attempts to formulate an equation expressive of apparently related biologic phenomena.

THE ESTIMATION OF SMALL AMOUNTS OF CARBOHYDRATES BY BACTERIAL PROCEDURES

STUDIES IN BACTERIAL METABOLISM, LXVI

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An important observation by Pasteur¹ that the mold, *Penicillium glaucum*, would destroy the dextrose isomer of tartaric acid (ammonium tartrate), leaving the laevo isomer practically untouched, at least until the former was quantitatively destroyed, opened a new field in biochemistry, in which microbes may be used advantageously as chemical reagents. The fundamental researches of Emil Fischer² on the important group of carbohydrates added an extensive class of organic compounds in which microbic analysts may be utilized in a highly interesting manner. Fischer recognized the possibilities in this field. His work with Thierfelder³ on the fermentation of hexose sugars by pure yeast cultures focused attention on the significance of the relation between chemical stereoconfiguration and protoplasmic catabolism.

It is unfortunate that bacteriology was insufficiently advanced at the period when Fischer's interest was directed toward the biologic import of the asymmetric carbon atom to afford a more versatile group of organisms than the yeasts for experimentation. The multitude of subsequent studies on the same theme, in which bacteria have supplanted yeasts, lack Fischer's exceptional chemical background which is indispensable for success. Nevertheless, distinct advances have been made, particularly with reference to the microbic aspect of the problem. Two studies by Theobald Smith⁴ early in the history of bacteriology paved the way for later observations. The point of view of these investigations was unlike that of Fischer in that the structure of the sugars used as differential tests for microbic identification was not considered, although of course it was an essential feature. Smith made the significant discovery that pathogenic bacteria, which multiply in the tissues

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¹ Compt. rend., 1858, 46, p. 615.

² Untersuchungen über Kohlenhydraten und Fermente, 1884-1908, 1909.

³ Ber. d. deutsch. chem. Gesellsch., 1894, 27, p. 2031.

⁴ The Fermentation Tube, Wilder Quarter Century Book, 1895, p. 187; Notes on *Bacillus Coli* and Related Forms, Am. Jour. Med. Sc., 1895, 110, p. 283.

of the body rather than in the outside world, tend to conform in their general carbohydrophilic relations to those of the tissues themselves; that is to say, the pathogenic bacteria are distinctly less versatile in their attack on carbohydrates than the parasitic types, as a general rule.

Another group of experiments, extensive in the aggregate, has been devoted to an effort to classify bacteria on the basis of their respective abilities to utilize one or another selection of carbohydrate and carbohydrate-like substances. Such studies are statistical, rather than analytic, but they have served a useful purpose. They have shown that a number of carbohydrates, other than those exhibiting 3 or a multiple of 3 carbon atoms, are readily fermentable by some bacteria, thus modifying one of Fischer's ⁵ generalizations based on his studies with yeasts; and, also, that many of the possible carbohydrate configurations possess little or no value as diagnostic agents in bacterial identification.

Much of this work will require confirmation in the light of the important observation of Lobry deBruyn and van Ekenstein ⁶ that carbohydrates, particularly the aldose and ketose hexoses, undergo a rearrangement in slightly alkaline solutions, which results in equilibrium mixtures of three or more hexoses in place of the single original hexose. The methods of preparation of ordinary nutrient sugar mediums for bacterial use are, on the whole, theoretically favorable to the excitation of these changes. Consequently, the finer points of differentiation, when closely related sugars, such as glucose, fructose and mannose are used, must be redetermined under conditions where this readjustment of hexoses cannot occur.

The purity of carbohydrates used in microbiologic studies must be considered. In general, the specific optical rotation of the various sugars is a valuable quantitative factor for their identification. With the Bates variable brightness ⁷ modification of the Frick polariscope, extremely accurate determinations of the rotation of sugar solutions may be made. It will be remembered, however, that some carbohydrates, such as dulcitol, are optically inactive through internal compensation of right and left rotating groups. Furthermore, standards of precision of sufficient refinement to detect and identify small amounts of alien optically active substances are not available for all sugars. Thus, glucose and lactose rotate the plane of polarization 52.5 degrees

⁵ Untersuchungen über Kohlenhydraten und Fermente, p. 108.

⁶ Ber. d. deutsch. chem. Gesellsch., 1895, 28, p. 3078.

⁷ Bulletin, Bureau of Standards, 1907, 4, p. 461.

to the right under standard conditions. Glucose may be, and frequently is, a natural contaminant of lactose.⁸ It is quite clear that this glucose impurity of lactose cannot be detected with the polarimeter; it might even be unsuspected unless specifically sought for.

A discussion of chemical and physical standards of purity for sugars cannot be entered on at this time; it may be stated, however, that there appears to be a distinct place for relatively simple procedures which will permit a discrimination between optically active organic compounds. Bacteria are accurate appraisers of optical antipodes,⁹ in virtue of the fixed relation which appears to exist between the asymmetry of their protoplasm and the configuration of sugars they can utilize for their energy requirements. These relationships are of sufficient diversity among the bacterial types to permit of their application to the detection and identification of even small amounts of sugars. Bacteria, in other words, are potentially sugar analysts.

The basis for the conversion of microbic specificity of action on sugars into a diagnostic procedure depends on a fundamental principle of the metabolism of bacteria, namely, that any carbohydrate possessing the requisite stereoconfiguration to fit specific microbic protoplasmic asymmetry is utilized more readily for energy than nitrogenous protein derivatives¹⁰ when both are present. The adaptation of this principle to the present problem depends on the degree of refinement to which current methods may be shaped.

In general, the action of microbes on utilizable carbohydrates is manifested by an increase in the hydrogen-ion concentration of the medium in which they are developing. Some bacteria produce gaseous products from sugars in addition to the acidic changes. As changes in hydrogen-ion concentration are detectable and even measurable with a fair degree of accuracy by suitable chemical indicators, this procedure at once presents itself as a logical starting point.

Many chemical indicators are available, and two rather distinct uses may be made of them: First, a color change which shows that at least a minimum change in reaction toward the acid side has taken place, and, second, an accurate measure of the rate and extent of this change. The latter requires a graded series of solutions of known hydrogen-ion concentration with which the culture medium may be com-

⁸ Smith, Theobald: *Jour. Boston Soc. Med. Sc.*, 1897, 2, p. 236; Jones: *Jour. Infect. Dis.*, 1914, 15, p. 357.

⁹ Kendall: *Bacteria as Chemical Reagents*, *Jour. Chem. and Metall. Engin.*, 1921, 24, p. 56.

¹⁰ Kendall and Farmer: *Jour. Biol. Chem.*, 1912; 12, pp. 13, 215, 465. Kendall, Day and Walker: *Jour. Am. Chem. Soc.*, 1913, 35, p. 1201; 1914, 36, p. 1937.

pared from time to time. There is an unmistakable advantage in the latter alternative. This is quite clearly shown in a preliminary series of experiments which are omitted from this discussion because they were not sufficiently delicate to meet the requirements of the proposed procedure. Out of the experience gained in these studies, however, a method has been evolved which is simple, direct and quite rapid.

PROCEDURE

A medium is prepared containing 0.25% of neutral peptone solution¹¹ in tap water,¹² together with 10 cc.¹³ of a buffer solution containing enough M/5 disodium hydrogen phosphate and dihydrogen potassium phosphate, respectively, to bring the final reaction to P_H 6.8. About 6.8 cc. of the former and 3.2 cc. of the latter usually suffice. This is the basic nutritive substrate. It may be sterilized with steam in the autoclave, or by passage through suitable stone filters. The latter procedure is preferable. The medium is dispensed in 500 cc. flasks, adding about 300 cc. to each flask. A broad surface of fluid is exposed to the air, and this is advantageous.

The sugar solution to be tested is prepared carefully and accurately. In practice, exactly 1 gm. of the pure and dry sugar is introduced quantitatively into a 100 cc. graduated flask. Enough neutral distilled water is added to bring the sugar fully into solution; then the solution is made up to the mark and set aside until equilibrium is reached. This may take several hours. Bacteria do not grow in pure sugar solutions; therefore no change may be expected to occur from microbial development.¹⁴

At the proper time the standard sugar solution is filtered through a chemically clean, bacterially sterile stone filter, and the filtrate is collected in a sterile flask.

The microbe (or microbes) selected for the test are grown at least 3 successive days on agar slants (0.1% glucose may be added with advantage to the agar), transferring daily. By so doing the bacteria are rejuvenated, and grow with relative luxuriance and uniformity.

The test is performed in the following manner:

1. Inoculate a flask of the nutritive peptone medium¹⁵ with the desired microbe early in the morning.
2. Prepare dilutions of the 1% stock sugar solution with sterile neutral distilled water, using proper precautions.¹⁶
3. When the growth of the microbe is clearly discernible in the peptone medium, usually within 2 hours, the culture is distributed quantitatively, 40 cc. to a portion, in each of 7 sterile 100 cc. flasks. The flasks are then reinforced, each with 10 cc. of a sugar dilution. They contain, therefore, the following

¹¹ Neutralized asparagin solution of the same strength may be substituted with advantage where the less fastidious bacteria, as *Bacillus mucosus capsulatus*, are used.

¹² The dissolved salts found in ordinary tap water are of some obscure value.

¹³ To each liter.

¹⁴ Heat and alkaline water must be rigorously avoided. Many sugar solutions undergo unmistakable alterations in heated or alkaline solutions.

¹⁵ It is best kept in the incubator over night; as an alternate procedure, the medium may be heated to 37 C. immediately before inoculation. Growth of the microbe starts at once.

¹⁶ Advantage may be taken of decimal dilutions: Thus, 1 c.c. of a 0.1% dilution + 9 c.c. distilled water will of course give a 0.01% dilution. This is simpler than adding 1 c.c. of the 1% solution to 99 c.c. distilled water and approximately as accurate because the sugar solution is thoroughly distributed in each c.c. of the diluting fluid.

TABLE 1
STOCK SUGAR SOLUTION PREPARED WITH STERILE NEUTRAL DISTILLED WATER

	Stock Solution	Distilled Water	Diluted Solution Contains	
			Grams Sugar per C c.	Per cent Sugar per C c.
Dilution a.....	1 c c.	9 c c.	0.001	0.10
Dilution b.....	1 c c.	19 c c.	0.0005	0.050
Dilution c.....	1 c c.	39 c c.	0.00025	0.025
Dilution d.....	1 c c.	79 c c.	0.000125	0.0125
Dilution e.....	1 c c.	199 c c.	0.00005	0.0050
Dilution f.....	1 c c.	399 c c.	0.000025	0.0025

TABLE 2
CONTENTS OF FLASKS

	Medium	Distilled Water	Sugar Dilution	The Medium Contains	
				Grams Sugar	Percentage Sugar
Flask 1 ¹⁷	40 c c. bacteria	10 c c.	..	0.00	0.00
Flask 2.....	40 c c. bacteria	10 c c.	a	0.00020	0.02
Flask 3.....	40 c c. bacteria	10 c c.	b	0.00010	0.01
Flask 4.....	40 c c. bacteria	10 c c.	c	0.000050	0.005
Flask 5.....	40 c c. bacteria	10 c c.	d	0.000025	0.0025
Flask 6.....	40 c c. bacteria	10 c c.	e	0.000010	0.0010
Flask 7.....	40 c c. bacteria	10 c c.	f	0.000005	0.0005

¹⁷ This is the control.

TABLE 3
INGREDIENTS CONTAINED IN TUBES

Tube A (control) 1 c c. water.....	no sugar, peptone medium, bacteria
Tube B.....	sugar, 0.02%, peptone medium, bacteria
Tube C.....	sugar, 0.01%, peptone medium, bacteria
Tube D.....	sugar, 0.005%, peptone medium, bacteria
Tube E.....	sugar, 0.0025%, peptone medium, bacteria
Tube F.....	sugar, 0.0010%, peptone medium, bacteria
Tube G.....	sugar, 0.0005%, peptone medium, bacteria

TABLE 4
COMPARISON OF SUGAR CONCENTRATION IN TUBES CONTAINING ORGANISM WITH A SET
OF STANDARD SOLUTIONS CONTAINING HYDROGEN-ION CONCENTRATIONS
ORGANISM BACILLUS COLI I SUGAR LEVULOSE

Time	Tube A Control (No Sugar)	Sugar Concentration, Percentage per C c.				
		Tube B 0.02	Tube C 0.01	Tube D 0.005	Tube E 0.0025	Tube F 0.001
Start	P _H 6.9	6.9	6.9	6.9	6.9	6.9
2 hours	P _H 6.9	6.7	6.8	6.9	6.9	6.9
3 hours	P _H 6.9	6.5	6.6	6.6	6.7	6.9
4 hours	P _H 6.9	5.9	6.2	6.4	6.5	6.8
5 hours	P _H 7.0	5.8	6.1	6.4	6.6	6.9
6 hours	P _H 7.1	6.0	6.5	6.7	6.8	7.0

ingredients in common: 40 cc. of a peptone solution of reaction about P_H 6.8, and a uniform growth of a particular microbe. The individual contents are given in table 2.

4. Incubate the several flasks for 2 hours.

5. Remove exactly 5 cc. of medium from each of the several flasks, and place each portion in a tube suitable for hydrogen-ion measurement. The tubes will contain the ingredients given in table 3.

To each tube add 3 drops of brom thymol blue.¹⁸

Compare the color of each tube with a set of standard solutions containing graded hydrogen ion-concentrations. The observations are repeated at hourly intervals for a period of not less than 6 hours under ordinary conditions.

DISCUSSION

The sparing action of the levulose for the protein constituents of the peptone medium is quite clearly indicated by the gradual increase in the hydrogen-ion concentration in the various sugar mediums. It is, as might be expected from the theory,^{9,10} quantitatively different for each tube and dependent on the concentration of the sugar. In the higher amounts, the reaction falls away more rapidly from neutrality than in the lesser concentrations. As the number of bacteria is approximately equal in each tube at the start, from the conditions of the experiment, the values have considerable quantitative value. It should be remembered, however, that the tubes containing utilizable carbohydrate offer rather greater opportunity for rapid microbic growth than is the case in the control tube where utilizable carbohydrate is absent.

The return toward neutrality in the sugar tubes indicates that at the specified time, or thereabouts, the sugar has been used up, and the microbes are forced to derive their energy from the protein constituents of the medium. The products under this condition are basic, and the resultant development of alkalinity is a quantitative measurement of the accumulation of potential hydroxyl ions. The control tube clearly shows this gradual development of an alkaline reaction, which is progressive.

One-thousandth of 1% (one one-hundred-thousandth of a gram per c.c.) of levulose is the smallest amount of this sugar that will furnish enough hydrogen ions to change the reaction measurably under the conditions of the experiment. Experience has shown that this amount is in general the minimum, and it may be assumed, therefore, to represent approximately the limit of precision of this method.

¹⁸ If the reaction falls below P_H 6.4, brom cresol purple is used. Other indicators may be employed, but experience has shown that the 2 mentioned are satisfactory.

It is of course possible to interpolate between the smaller amounts of sugar, and thereby increase the probable accuracy of the smaller readings. A long series of determinations with a variety of sugars and many kinds of bacteria has shown quite definitely that changes in reaction may confidently be expected in from 2 to 4 hours, and that 6 hours usually suffice to expose the full intensity of the reaction in the weaker sugar solutions. If greater concentrations are used, more time must be allowed for the bacteria to reach their maxima in hydrogen-ion concentration.

The experiment quoted, which is typical of many scores of similar studies, indicates that the method herein described is one of rather general application. It is of sufficient delicacy to permit of the detection of minute amounts of carbohydrate in solution through the proper use of bacterial reagents. It seems improbable that less than one one-hundred-thousandth of a gram of sugar in solution per c c. of medium can be detected with assurance by the procedure.

CARBOHYDRATE IDENTIFICATION BY BACTERIAL PROCEDURES

STUDIES IN BACTERIAL METABOLISM, LXVII

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The early history of bacterial cultivation in various organic mediums is naturally a catalogue of trial and rejection of readily available substances. Many studies on this theme have been recorded in which carbohydrates, carbohydrate-like substances, and carbohydrate derivatives have been added to cultural mediums to increase their nutritive value. Incidentally, considerable information has been gained concerning the uses of these substances for bacterial differentiation. This point of view, however, has its origin in the microbe rather than the medium; the significance of the results has been bibliographic rather than biologic. It is obvious that such should have been the case, because hitherto interest has centered in microbic differentiation rather than in biochemical relations. Indeed, bacteriology arose and emerged as a science under the patronage of pathology at a period when morphology and classification dominated both fields.

The pioneer and fundamental observations of Escherich¹ on the intestinal bacteria were the first to suggest relationships between nutritive substances and microbic activities. Many of the procedures for bacterial study in common use even today were foreshadowed in this remarkable work.

The enthusiasm which followed the discovery of the inciting agents of tuberculosis, anthrax, cholera, typhoid, diphtheria, tetanus, and other infections naturally withdrew attention from Escherich's studies and focused the attention of the medical world on microbic etiology. The production of curative serums for diphtheria and tetanus gave new impetus to the study of bacteria from the point of view of remedial antibodies. In spite of repeated efforts, however, soluble toxins could not be found among the products of growth of tubercle, cholera, typhoid and many other microbes; and, consequently, antitoxic serums could not be prepared. Nevertheless, this was an important period, and out of the great volume of experiments there arose a new science, immunology, which contemplates those complex, reciprocal relations which exist between host and microbe in infection.

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¹ Darmbakterien des Säuglings, 1886.

Meanwhile the workings of bacteria were revealed in new fields. Agricultural science was enriched with the unfolding of that remarkable symbiosis of nitrogen-fixing bacteria and the leguminous plants whereby the soil is refurnished with nitrogen. This humble microbe has stood between life and nitrogen starvation for centuries. The industries also have awakened to the part played by bacterial life in some of the most venerable processes known to mankind. The retting of flax, the tanning of leather, the manufacture of vinegar, the preservation of milk in the desert, the preservation of food for cattle (ensilage), and, more recently, the manufacture of synthetic rubber—all these are isolated instances of the utilitarian activities of bacteria.

The older conception of bacteria as implacable enemies of mankind is giving way to a recognition of their beneficent participation in many essential life processes. Also, and this is important, the idea is gaining ground that bacteria are living chemical reagents. The control of microbic action in the human body and in the arts and sciences will depend on the development of chemical knowledge. There seems to be no reasonable doubt that microbic activity, properly directed and controlled, will furnish an economical and tireless source of energy utilizable in a multitude of fields of interest and importance to the human race.

At the present time the application of serums and of various organic food substances to the diagnosis and identification of bacteria and to the results of their action are so generally recognized that they may be considered as integral parts of the science of bacteriology.

The time has come when the reverse procedure—the use of bacteria and of their products in the diagnosis of certain groups of organic substances—may be considered with advantage. This is not an entirely unexplored field. The Schick reaction furnishes valuable information concerning the vulnerability of the human body to the toxin of the diphtheria bacillus. *Bacillus coli* and some other bacteria will identify tryptophan in suitable mediums through the formation of indol, which gives a characteristic color reaction with nitrous acid. The presence of a small amount of glucose as a normal constituent in normal milk was first indicated by bacterial reactions.

The utilization of bacteria as chemical reagents in the field of carbohydrate chemistry is a new but highly interesting one for experiment and speculation. Living protoplasm and carbohydrates have peculiar and significant interrelations. Thus, d-glucose appears to be the one sugar which can be oxidized with advantage to furnish energy in the human body. Other sugars that may form part of the food of man

are changed to glucose in ways not clearly understood prior to their introduction into the tissues. The lactating mammary gland secretes lactose, a sugar not available as such for the energy requirements of the tissues. This appears to be advantageous. If glucose were secreted, the maternal tissues would be in competition with the nursing for the carbohydrate of the milk.

One of the unsolved problems of lactation is the mechanism through which galactose is formed from glucose, prior to the compounding of the lactose. Certain tissues of the nervous system contain considerable amounts of a sugar provisionally identified as galactose. As galactose is not readily oxidized in the body for energy, it would appear to be a more satisfactory substance for structural purposes than glucose, which would of course always be potentially available for fuel to the detriment of its structural stability. Many other problems that cannot be even mentioned at this time indicate the wealth of material awaiting elucidation in this fascinating and important field of protoplasmic asymmetry and stereoconfiguration of organic substances.

The immediate object of this communication, aside from a mere suggestion of the range of possibilities of microbic chemistry, is a limited discussion of the application of bacteria to the identification of closely related carbohydrates. There are many angles to this problem. The hydrolysis of bioses, trioses,² glucosides, starches, and gums, leaves fragments which are frequently difficult to identify, particularly with energetic chemical reagents which may induce secondary changes of unknown extent in these fragments. Also, many naturally occurring carbohydrates are associated with traces of other carbohydrates which may be normal constituents, or the result of methods of preparation.

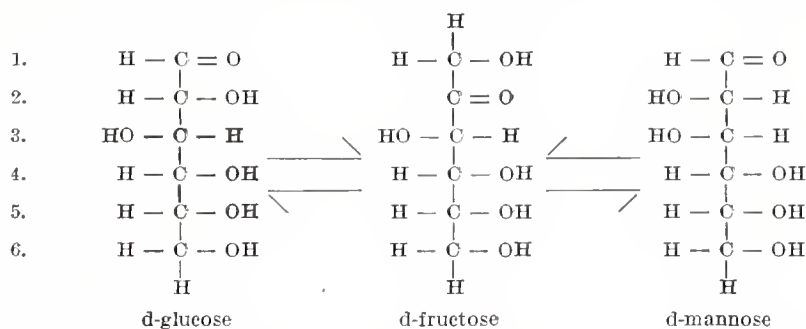
Bacteria are delicate reagents. Their action is chiefly on such of these fragments as happen to possess a stereoconfiguration which can be utilized for energy by the protoplasm of the microbe. The remainder is left intact. It follows that a standard series of microbes offers a clear-cut method of identification or separation of one or more sugars from an unknown mixture. The serial application of this principle would theoretically permit of the identification of a number of simpler sugars resulting from the cleavage of an unknown complex carbohydrate. Also, small amounts of impurities in presumably pure sugars may be detected by the same process.

² There seems to be no suitable generic term for sugars compounded of 2 hexose sugars, such as maltose, lactose or saccharose. Maquenne (*Les sucres et principaux dérivés*, 1900) uses the term *biose* and *triose* to indicate, respectively, two or three members of the hexose series, compounded with the loss of H and OH. It is clear that the term *biose* is more strictly applicable to substances such as glycol or glycolic aldehyde.

Before definite application of bacteria to carbohydrate identification can be taken advantage of, however, it is obvious that certain standards must be established. Two independent factors are involved: First, a set of carbohydrates of unimpeachable purity, suitably made up into nutrient mediums, and, second, a library of microbes to act on these carbohydrates in known and constant ways.

The basis for the observations presented herewith was a set of carbohydrates of great purity, obtained from the Bureau of Chemistry of the Department of Agriculture in Washington.³ The bacteria were taken from the library of microbes of the Medical School. The cultures have been kept on artificial mediums for many months. Their purity was thoroughly established, and the types exhibiting important diagnostic differences were investigated many times to establish the fact beyond reasonable doubt.

A detail of great importance in the bacterial study of carbohydrates, frequently overlooked, is the change which sugars containing 6 carbon atoms may undergo in mediums of even a slightly alkaline reaction.⁴ The evidence on which this change is based will be discussed in another communication. Suffice it to state that a 6 carbon sugar, such as d-glucose, placed in a slightly alkaline solution, undergoes a rearrangement of its H and OH atoms around the carbon atom next in line to the aldehyde group and the first carbon atom as well, in such a manner as to leave an equilibrium mixture containing at least d-glucose, d-fructose, and d-mannose. Other substances, chiefly organic acids, are formed at the same time. The relations are expressed in the following diagram:



³ Dr. Carl Alsberg, Dr. W. G. Campbell, Chiefs of the Bureau, Dr. Walton and Dr. LaForce, of the Carbohydrate Division, prepared these sugars. This work would have been impossible without this contribution of invaluable material and most cordial support.

⁴ Lobry de Bruyn and Van Ekenstein: *Rec. trav. chim. de Pays Bas*, 1895, 14, pp. 156, 203; 1896, 15, p. 92; 1897, 16, pp. 257, 262, 274; *Ber. d. deutsch. chem. Gesellsch.*, 1895, 28, p. 3078.

Similarly, d-galactose undergoes a change resulting in an equilibrium mixture (together with certain other substances) of d-galactose, d-talose, and d-tagatose.⁵

It must be obvious that the reaction of the medium in which 6 carbon sugars are dissolved must be carefully determined. As the usual initial reaction of cultural mediums is slightly on the alkaline side, older descriptions of reactions involving these sugars must be regarded with some suspicion.

In all the experiments recorded here, the mediums were adjusted to a reaction of P_H 6.8, or even more acid. Also, concentrated solutions of the sugars were sterilized apart by filtration through stone filters. The proper amounts were then added to the mediums. The action of both $O H$ -ions and of heat was thus avoided. The nitrogenous basis for the medium was meat extracted with water in the usual manner, freed from muscle sugar and reinforced with 0.5% peptone. The reaction was corrected to approximately P_H 6.8. One per cent. agar and 5 c.c. of the Andrade indicator⁶ per liter completed the stock nutrient medium, which was practically colorless when properly prepared. This was dispensed in small test tubes, and at the proper time enough sugar solution was added to make a final concentration of 0.5%. Incubation to determine sterility was practiced, and inoculations were made with a needle in the usual manner, but with 3 separate stabs to each tube. Cultures transferred daily for 3 successive days were employed as inocula.

A change in reaction toward the acid side was indicated by the appearance, within 24 hours, of a brilliant red. If the reaction remained unaltered or became progressively alkaline, no color change was of course detectable. As the utilization of carbohydrate for energy is associated, so far as is known, with acidic products, this Andrade indicator method was satisfactory for the detection of microbic action on the various sugars.

Experiments showed that as small an amount of sugar as 0.005% per c.c. and often 0.0025%, gave a distinct but transient reddening of the medium.⁷ The change in reaction was frequently noticeable within 4 hours after inoculation if the conditions for microbic growth

⁵ Nef (Liebig's Ann., 1907, 357, p. 294; 1910, 376, p. 1) believed that 8 or more sugars were in equilibrium when d-glucose or d-galactose was placed in solution in an alkaline fluid.

⁶ Andrade: Jour. Med. Res., 1906, 14, p. 551.

⁷ This corresponds to 0.00005 or 0.000025 gm., respectively, of sugar per c.c. of medium.

were favorable. For purposes of identification, however, the larger amount of carbohydrate—0.5%—was used.

Twenty-five carbohydrates, including glycol, glycerol, pentose sugars and alcohols, hexose sugars and alcohols, bioses and trioses, glucosides and starches were studied with about 200 different cultures. Much of the information will be presented in another communication. The salient facts relating to the identification of the more common sugars—glucose, fructose, mannose, galactose, lactose and sucrose—are presented herewith for the purpose of indicating the method of procedure for identifying carbohydrates by microbial reagents. Only those common bacteria which exhibit differential diagnostic value for this limited series of sugars are mentioned.

A brief survey of this table of fermentation reactions, in which the symbol + indicates fermentation⁸ will indicate the diagnostic use

TABLE 1
FERMENTATION REACTIONS

Microbe Reagent	Sugar					
	Glucose	Fructose	Mannose	Galactose	Lactose	Saccharose
<i>B. proteus</i>	+	—	—	+	—	+
<i>Mic. tetragenus</i>	+	+	—	+	—	—
<i>Vibrio cholerae</i>	+	+	—	+	+	+
<i>Vibrio</i> of Finkler and Prior.....	+	+	+	—	—	+
<i>B. typhosus</i>	+	+	+	+	—	—
<i>B. coli</i> I.....	+	+	+	+	+	—
<i>B. coli</i> II.....	+	+	+	+	+	+

of the several bacteria, and will also suggest how impurities may be sought for in sugar solutions, provided the amount of impurity equals or exceeds about 0.00003 gm. per c. c. of medium.

Thus, in a sucrose solution⁹ containing enough glucose or fructose to give an acid reaction (0.00003 gm. of glucose per c. c. of culture medium or more), *Bacillus typhosus* would give a distinct reddening of the medium even though it does not ferment saccharose. In a similar manner, a small amount of glucose or galactose could be detected as an impurity in lactose, because *Bacillus typhosus* would ferment both the glucose and the galactose, leaving the lactose unaltered.

A distinction could be made between the glucose and the galactose even, because the *vibrio* of Finkler and Prior used in these tests fer-

⁸ No attention is paid to the formation of gas in the table—a change indicating an increase in H-ions alone is recorded.

⁹ Broth mediums must be used in place of agar mediums for this purpose.

ments glucose, but neither galactose nor lactose.¹⁰ An additional refinement, whereby the galactose could be reidentified, would be simply accomplished by filtering the medium through a stone filter, after the glucose has been used up by the vibrio of Finkler and Prior, and after readjusting the reaction to the proper point by the addition of alkali; then inoculating with *Bacillus typhosus* or any of the galactose fermenting bacteria that do not utilize lactose. An acid reaction would appear if the amount of galactose were sufficient to give a color reaction with the indicator. Mannose and fructose could be eliminated as possibilities by a serial use of the bacteria which is perfectly obvious.

Glucose is the only carbohydrate thus far encountered which is fermented by all microbes that ferment any sugar at all.

This utilization of a library of carefully standardized microbes for the identification of water-soluble carbohydrates seems to be almost unlimited. The determination of carbohydrates, both as they occur naturally and of their cleavage products, the detection of small amounts of impurities in various sugars, and the separation of carbohydrates by microbic means open up a field for the sugar chemist which has not been explored hitherto, or at least has not received the recognition as a scientific method which its possibilities would seem to indicate.

¹⁰ It will be noted that many of the diagnostic strains are pathogenic organisms. This objection may be avoided by studying a sufficient number of nonpathogenic bacteria to find adequate substitutes. The principle, not the details, is the primary objective for consideration here.

THE MEASUREMENT OF CARBOHYDRATE MIXTURES BY BACTERIAL PROCEDURES

STUDIES IN BACTERIAL METABOLISM, LXVIII

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One of the important biochemical reactions of value for the differentiation of various pathogenic bacteria is that one elicited by dysentery bacilli and some others in milk. The addition of an indicator to the culture assists materially in the recognition of the details. The early studies on the paratyphoid group by Achard and Bensaude¹ and others, included a description of this reaction in milk, in which an initial acidity followed by an alkalinity was recognized as an important differential point, characteristic for certain members of the group. The chemistry of this reaction was unknown. Subsequent investigations have revealed the presence of a small amount of glucose, a normal constituent of milk unsuspected by the chemists.²

The first evagation is a change in reaction toward the acid side, and this is followed in 2 or 3 days by a recession in the acidity to the neutral point, or even beyond to the alkaline side. Not every sample of milk reacts in this manner, even with the same culture, under apparently parallel conditions. For this reason many bacteriologists are inclined to discount the value of this milk culture reaction on the basis of some irregularity in its occurrence. Without entering into a discussion, it may be stated that the observed irregularities are due in no small degree to the age of the milk used for the test. Some of the reasons will be clear when the chemistry of the reaction is unfolded.

From the published analyses of milk, it is quite clear that at least 4 non-nitrogenous compounds are available from which an acid reaction may be divided. Two of these are carbohydrates, glucose (0.1%) and lactose (4.6%); two are not of the carbohydrate group, namely, the fatty acids and glycerol. Bacterial development in milk, therefore, may lead to an acid reaction either by the fermentations of glucose, lactose, or both; by the cleavage of neutral fat by a bacterial lipase (or

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¹ Soc. Méd., sér. 3, 1896, 13, p. 679.

² Theobald Smith: Jour. Boston Soc. Med. Sc., 1897, 2, p. 236. Jones: Jour. Infect. Dis., 1914, 15, p. 357.

esterase) with the coincident liberation of free fatty acid, or by the fermentation of the glycerol after its liberation from the fat molecule.³

As the glucose does not exceed 0.1% under normal conditions, this small amount of sugar gives rise to a slight, transient acid reaction which tends to return to neutrality, or the alkaline side, if the lactose is unattacked or if a lipase is not included in the enzyme equipment of the microbe.

The principles involved in the transient acid reaction in milk have several important phases which bring together the fields of biologic chemistry, carbohydrate chemistry and bacteriology. For the biologic chemist, the bacteria are important in that through their action a new and unsuspected constituent of milk has been revealed. To the carbohydrate chemist, the microbe offers new and refined methods for the detection and determination of carbohydrates in complex mixtures. A reaction of diagnostic value elicited in a natural medium, such as milk, is of obvious interest to the bacteriologist.

The detection and determination of organic compounds in complex mixtures in general is a field of peculiar interest to the sugar chemist. The multitude of carbohydrates produced in the vegetable kingdom particularly has led to much fruitful work, some of which is of fundamental importance in the domain of science. The complexity of the associated compounds in biologic mixtures containing sugars, however, renders the quantitative separation of the latter somewhat of a problem. Also, there is the frequent possibility of change or loss attending such manipulations. Herein lies a field for microbic exploration. Some of the possibilities have been mentioned previously.⁴

The procedure to be followed varies with the nature of the associated substance. In milk, all the ingredients are essentially nutritional, but the distinction between the carbohydrate content on the one hand and the nitrogenous and lipoidal substances on the other hand, is sharply defined by the nature of the reactions induced by specific microbes. Thus, bacteria that utilize glucose but not lactose produce an acid reaction up to the point of exhaustion of the simpler sugar. Those that ferment lactose cause a progressive acidity, which usually leads to coagulation of the casein.

Milk, however, offers some inexplicable peculiarities. *Bacillus coli*, for example, produces gas as well as acid from the fermentation of

³ It must be recognized also that acidic products may be formed from the bacterial putrefaction of casein; in this event the presence of a soluble proteolytic enzyme may be frequently detected. The microbe would not, however, attack the protein if it could use either the glucose or lactose, at least until the latter were used up.

⁴ Kendall: *Bacteria as Chemical Reagents*, Jour. Chem. and Metall. Engin., 1921, 24, p. 56. Kendall and Yoshida: *Study LXVI*, J. Infect. Dis., 1923, 32, p. 355.

lactose in ordinary nutrient fermentation mediums containing this sugar. In milk no gas is formed unless some proteolytic organism as, for example, certain strains of *B. mesentericus*, are allowed to act first.⁵ These observations are introduced to indicate the complexity that may arise in the solution of certain problems relating to the detection and estimation of carbohydrates in complex organic menstrea by microbic agencies.

Returning to the discussion of the detection of small amounts of carbohydrates, either alone or in association with other carbohydrates, the first step to be considered is the probable nature of the smaller sugar. This may be surmised in many instances. Thus, in saccharose, either glucose or to a lesser degree, levulose, may be confidently looked for; in lactose, glucose, or galactose. From more complex mixtures of carbohydrates, glucose must be ruled out.

Many of the rarer sugars and the alcohols derived from them are of value principally as diagnostic reagents for bacteria. The use of these sugars for bacterial differentiation depends on the two fundamental principles of the specific relationships between stereoconfiguration of sugars and corresponding asymmetry of bacterial protoplasm, and the sparing action of utilizable carbohydrates (that is, carbohydrate whose configuration can be burned by the bacterial substance for energy) for protein.

One additional feature deserves mention, namely, that certain types of carbohydrates, as those containing 6 carbon atoms, are prone to undergo material change in alkaline solution, a change of such magnitude that several sugars may exist in equilibrium where one existed formerly.⁶ It is necessary, therefore, to perform experiments on such sugars in mediums containing an initial slight excess of H-ions.

The detection of small amounts of sugars, alone or in the presence of larger amounts of other carbohydrates, is not a matter of great difficulty. The essential reagents are, first, a set of microbes of known fermenting powers; second, a set of carbohydrates of known purity; third, a set of standard phosphate solutions of known hydrogen-ion concentration.⁷ If the suspected carbohydrate is in considerable concentration—one-half to several per cent.—the regular test for fermentation may be performed, using a suitable nitrogen-containing medium as a basis. If the unknown carbohydrate is present in very small

⁵ Kendall: Boston Med. & Surg. Jour., 1910, 163, p. 322.

⁶ Lobry de Bruyn and van Ekenstein: Ber. d. deutsch. chem. Gesellsch., 1895, 28, p. 3076.

⁷ Kendall and Yoshida: Study LXVI, J. Infect. Dis., 1923, 32, p. 355.

amount, recourse to the measure of the H-ion must be practiced. The general procedure is explained in detail in an earlier communication.

The identification of the carbohydrate requires a careful study of its fermentability by the standard strains of bacteria described in the preceding study.⁸ If two carbohydrates are simultaneously present, the process is more complicated. The following schematic table, (table 1), however, illustrates graphically how the determination may be made with the bacterial reagents.

The method herein described, together with the quantitative measurements of the H-ion concentration by the various microbes described in previous studies,⁴ furnishes a procedure which permits of the proxi-

TABLE 1
IDENTIFICATION OF CARBOHYDRATE WITH BACTERIAL REAGENTS

Microbe Reagent	Sugar					
	Glucose	Fructose	Mannose	Galactose	Lactose	Saccharose
<i>B. proteus</i>	+	—	—	+	—	+
<i>Mic. tetragenus</i>	+	+	—	+	—	—
<i>B. mesentericus</i>	+	+	—	—	—	—
<i>Vibrio cholerae</i>	+	+	—	+	+	+
<i>Vibrio</i> of Finkler and Prior	+	+	+	—	—	+
<i>B. typhosus</i>	+	+	+	+	—	—
<i>B. coli</i> I.....	+	+	+	+	+	—
<i>B. coli</i> II.....	+	+	+	+	+	+

The sign + indicates an increase in the H-ion concentration of the medium, and therefore fermentation (utilization for energy) of the sugar. The sign — indicates an increase in the O H-ion concentration, due to action on the protein constituents of the medium for energy, and therefore no fermentation.

mate analysis of unknown sugar mixtures. A concrete example will be illustrative of the procedure.

Accurately prepared sterile 1% solutions of d-glucose, fructose, mannose, galactose, lactose and saccharose were prepared in neutral distilled water, and added to the nutrient nitrogenous medium described in Study LXVI in such amounts that the final concentrations of each sugar were exactly 0.02, 0.01, 0.005, 0.0025, 0.001, and 0.0005%, respectively. The mediums were inoculated with pure standard cultures of *B. proteus*, *B. mesentericus*, and *B. coli* I, respectively. Incubation was practiced for 4 hours, and then the H-ion concentration was determined for each dilution of sugar with each organism.⁹ For convenience, only the 4-hour determinations are recorded; it is advisable to make

⁸ Kendall: Study LXVII, *ibid*.

⁹ Footnote 4, second part.

readings at 3, 4, 5, 6 and 8 hours with corresponding dilutions to sugars to obtain the rate and extent of the H and OH ion changes:

TABLE 2
H-ION CONCENTRATION AFTER FOUR HOURS OF INCUBATION OF *B. PROTEUS* III

Organism: <i>B. proteus</i> III Concentration of Sugar, Percentage	H-ion Concentration; Time, Four Hours					
	Glucose	Fructose	Mannose	Galac- tose	Lactose	Saccha- rose
Control Ph.....	6.8	6.8	6.8	6.8	6.8	6.6
0.02%.....	5.3	6.8	6.8	5.3	6.8	5.3
0.01%.....	6.2	6.8	6.8	6.0	6.8	5.9
0.005%.....	6.3	6.8	6.8	6.5	6.8	6.3
0.0025%.....	6.5	6.8	6.8	6.6	6.8	6.4
0.001%.....	6.7	6.8	6.8	6.7	6.8	6.5
0.0005%.....	6.8	6.8	6.8	6.8	6.8	6.6

It is clear that *Bacillus proteus* ferments glucose, galactose and saccharose; it fails to utilize fructose, mannose and lactose for energy.

TABLE 3
H-ION CONCENTRATION AFTER FOUR HOURS OF INCUBATION OF *B. MESENTERICUS*

Organism: <i>B. mesentericus</i> Concentration of Sugar, Percentage	H-ion Concentration; Time, Four Hours					
	Glucose	Levulose	Mannose	Galac- tose	Lactose	Saccha- rose
Control Ph.....	6.9	7.0	6.9	6.8	6.9	6.9
0.02%.....	5.3	5.3	6.9	6.8	6.9	6.9
0.01%.....	6.1	6.0	6.9	6.8	6.9	6.9
0.005%.....	6.5	6.5	6.9	6.8	6.9	6.9
0.0025%.....	6.6	6.7	6.9	6.8	6.9	6.9
0.001%.....	6.8	6.8	6.9	6.8	6.9	6.9
0.0005%.....	6.9	7.0	6.9	6.8	6.9	6.9

Bacillus mesentericus ferments glucose and levulose, but fails to derive energy from mannose, galactose, lactose or saccharose.

TABLE 4
H-ION CONCENTRATION AFTER FOUR HOURS OF INCUBATION OF *B. COLI* I

Organism: <i>B. coli</i> I Concentration of Sugar, Percentage	H-ion Concentration; Time, Four Hours					
	Glucose	Levulose	Mannose	Galac- tose	Lactose	Saccha- rose
Control Ph.....	6.7	7.0	6.7	7.0	6.8	7.1
0.02%.....	5.3	5.5	5.3	5.6	5.3	7.0
0.01%.....	6.0	6.1	6.0	6.1	5.9	7.0
0.005%.....	6.3	6.4	6.4	6.5	6.5	7.0
0.0025%.....	6.5	6.6	6.5	6.6	6.6	7.0
0.001%.....	6.6	6.9	6.6	6.9	6.7	7.0
0.0005%.....	6.7	7.0	6.7	7.1	6.8	7.1

Bacillus coli ferments the four hexoses and lactose as well; saccharose is unattacked.

With these data as a starting point, indicative of the specificity and intensity of reaction of the several microbes on the selected list of sugars, the details of an actual experiment may be cited in which the identity and proximate measures of two sugars were determined.

A 1% lactose solution was intentionally contaminated with a 0.1% solution of glucose. This was compounded in the following manner: One gram of pure dry lactose was placed in a 100 c.c. volumetric flask. One gram of pure dry glucose was placed in a similar flask. The latter was made up to the mark with neutral distilled water, and after equilibrium was reached, exactly 10 c.c. of the glucose solution were placed in the lactose flask. This was made up to the 100 c.c. mark with the requisite amount of neutral distilled water, and the mixture of sugars allowed to reach solution equilibrium. Sterilization was accomplished by passing the mixture through a clean stone filter.

The first step in the actual test is to diagnose the sugars in the mixture. To do this, a set of tubes, each containing 4 c.c. of the regular nitrogenous, sugar-free nutrient medium receives 1 c.c. of the sugar solution. Each tube contains a final concentration of 0.2% lactose (0.002 gm. per c.c. of medium) and 0.02% glucose (0.0002 gm. per c.c. of medium). A control tube containing 1 c.c. of water in place of the sugar solution is added to the set. The several tubes are inoculated with cultures of *Bacillus proteus*, *mesentericus* and *coli*, respectively.¹⁰ After four hours' incubation the reactions were as follows:

Control	P _H 6.9
<i>Bacillus proteus</i>	6.9
<i>Bacillus mesentericus</i>	6.7
<i>Bacillus coli</i>	5.2

The larger sugar is clearly not saccharose, otherwise *B. proteus* would have formed enough acid to give a P_H value approaching that of *B. coli*. It is not levulose; otherwise *B. mesentericus* would produce a much higher hydrogen-ion concentration. Mannose and galactose are ruled out for similar reasons. Lactose alone, according to the tabulated information, would give this result.

Following a similar line of reasoning, the smaller sugar is neither mannose, levulose nor galactose; otherwise *B. proteus* and *B. mesentericus* would not give approximately equal H-ion concentrations, each less

¹⁰ A larger series of bacteria is required for unknown mixtures. The example herein described is abbreviated to save space.

than the control value. In other words, were the smaller sugar levulose, *B. mesentericus* but not *B. proteus* would show an increase in acidity. Mannose would not differ in reaction from the control and galactose would show an increase in H-ion concentration in the tube infected with *Bacillus proteus* but not in the tube containing *B. mesentericus*, if the amount of the sugar were as indicated—0.02%.

The final step in the process is the estimation of the amounts of the 2 sugars. To accomplish this, 2 sets of tubes are set up for the lactose estimation, and 2 sets for the glucose determination. One set of each series contains graduated amounts of lactose and of glucose, respectively. The other set contains the unknown mixture in graduated amounts of the same magnitudes.

For ordinary purposes, dilutions in multiples of 10 are well suited for the purpose. Thus, the known lactose set, containing accurately measured amounts of the sugar in the following percentages per c.c. of medium would be employed, 0.02, 0.01, 0.005, 0.0025, 0.001, 0.0005. An exactly parallel series of the unknown lactose set is prepared. Both sets are then infected with *Bacillus coli*, with the following results after 4 hours' incubation:

TABLE 5
RESULTS OF ESTIMATIONS OF AMOUNTS OF TWO SUGARS

Inoculum <i>B. coli</i>	Pure Lactose Solution		Unknown Lactose Solution	
	Concentration	P _H	Dilution	P _H
Control.....	0	6.9	0	6.9
	0.02%	5.3	0.02%	5.3
	0.01%	6.1	0.01%	6.0
	0.005%	6.4	0.005%	6.3
	0.0025%	6.6	0.0025%	6.5
	0.001%	6.7	0.001%	6.7
	0.0005%	6.9	0.0005%	6.9

The close approximation between the acid curves of the known lactose and the suspected lactose solution points to an equal concentration of the two sugars. As the known solution is 1% in the undiluted state, it is probable that this figure represents the concentration of the unknown solution quite accurately.

In a similar manner, the unknown smaller sugar is compared with a glucose solution of known strength. As the glucose in the unknown mixture is 10 times as dilute as the lactose solution referred to above, the detectable acidity of the unknown glucose solution would cease to be apparent at about the level of the 0.01% known solution of glucose,

which is by definition 10 times stronger. A second series of known and unknown glucose dilutions must be prepared of such strengths that the amounts between 0.05 and 0.001% in the culture tubes differ on a smaller scale. A careful study of the second series will furnish data for a fairly accurate estimation of the concentration of the smaller sugar in the original sample.

CARBOHYDRATE CONFIGURATION AND BACTERIAL UTILIZATION

STUDIES IN BACTERIAL METABOLISM, LXIX

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It has been recognized for many years that various carbohydrates and derivatives of carbohydrates possess value for the cultural differentiation of yeasts and bacteria. It is not generally recognized, however, that the value of carbohydrates and their derivatives for this purpose depends on two distinct principles, namely, the sparing action of utilizable carbohydrate for protein, when both are present simultaneously in available form,¹ and also that the carbohydrate, to be utilizable, must possess a stereoconfiguration which is compatible with a corresponding asymmetry of the protoplasm of the microbe. Emil Fischer expressed this latter relationship as that of a "key fitting its particular lock" in his well-known study of the action of yeast enzymes upon sugars and glucosides.²

The first important systematic study of carbohydrate utilization from the point of view of stereoconfiguration was made by Fischer and Thierfelder,³ nearly 30 years ago. This was a most important investigation because Fischer, the great master of carbohydrate chemistry, procured various carbohydrates of known composition and purity for the work. Unfortunately, the bacteria, which are far more versatile as a group than the yeasts in their carbohydrate relations, were not tried at this time. The restraints imposed by the restricted carbohydrate activity of the yeasts limited the results of these investigations to a few general facts. One of these was the mutual fermentability of certain natural groupings of hexose sugars, such as glucose, fructose, and mannose, if one of the group was found to be fermentable.

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¹ Kendall and Farmer: *Jour. Biol. Chem.*, 1912, 12, pp. 13, 19, 215, 219, 465. Kendall, Day and Walker: *Jour. Am. Chem. Soc.*, 1913, 35, p. 1201.

² *Ztschr. f. physiol. Chem.*, 1898, 26, p. 60.

³ *Ber. d. deutsch. chem. Gesellsch.*, 1894, 27, p. 2031.

Lobry de Bruyn and van Ekenstein⁴ and Armstrong⁵ added a chemical background to this tendency toward group fermentation by pointing out the possibilities of a mutual transformation of the members within the group by a common enolization.

Armstrong⁵ later gave utterance to the suggestion that certain microbes might in fact bring about this enolization first, and then act on the common link (enol) between the members of the homologous sugar group. Yeasts indeed are quite commonly found to be fermenters of all the members of such a natural sugar group if they can ferment one member, and conversely it is apparently unusual to encounter pure yeast strains which can ferment one but not all members of two or more natural groups.³

There is some evidence to indicate that a similar limitation of fermentability to members of a naturally occurring group of hexose sugars is not uncommon among bacteria.⁶ Subsequent studies with a larger selection of bacteria, however, show quite clearly that the mutual fermentability theory does not hold for all.⁷ Thus, certain active microbes, such as *Bacillus proteus* and *Cholera vibrio*, ferment only glucose of the glucose-fructose-mannose series. Apparently fructose and mannose do not present a stereoconfiguration compatible with the asymmetry of the protoplasm of these bacteria. Judging from the glucose reaction, however, it would appear that neither *Bacillus proteus* nor *Cholera vibrio* can incite any change in either d-fructose or d-mannose, under the conditions which prevail in the usual bacterial experiment, which leads to the formation of a fermentable derivative of these sugars. Galactose of the galactose-talose-tagatose series is also fermented by these two microbes, but it cannot be stated that talose and tagatose are fermentable by them, because these sugars are unavailable.

The two best known of the naturally occurring groups found in the hexose series, in which d-glucose and d-galactose are the more commonly occurring respective members, include d-glucose, d-fructose and d-mannose on the one hand; d-galactose, d-talose and d-tagatose, on the other hand. If a member of either group, for example d-glucose, is dissolved in water having a slight excess of OH ions, a series of changes soon takes place which results in a fall of optical rotation to

⁴ Ibid., 1895, 28, p. 3078.

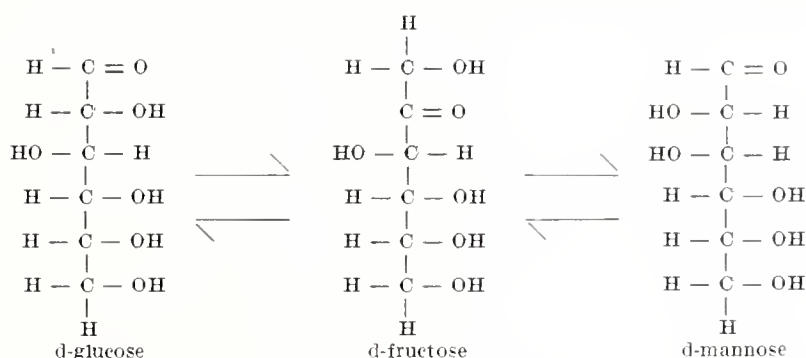
⁵ Simple Carbohydrates and Glucosides, 1912.

⁶ Kendall, Day, Walker and Ryan: Jour. Infect. Dis., 1919, 25, p. 189.

⁷ Kendall: Bacteria as Chemical Reagents, Jour. Chem. and Metall. Engin., 1921, 24, p. 56. Kendall and Yoshida: Jour. Infect. Dis., 1923, 32, p. 355.

about the neutral point, the appearance of acidic substances, including considerable lactic acid,⁸ and an equilibrium mixture of d-glucose, d-fructose and d-mannose. The sum of the 3 members, however, is apparently less than the amount of the sugar originally present. Some of this loss is accounted for by the acidic substances. Sunlight acts in a somewhat similar manner on sugar solutions.⁹

The 3 sugars, d-glucose, d-fructose and d-mannose are very much alike, as the diagram indicates.



The d-galactose, d-talose, d-tagatose series shows corresponding differences in the arrangement of the H and OH groups around the asymmetric carbon atoms. Even so slight a rearrangement as the reversal of the H and OH around the carbon atom next the aldehyde carbon in mannose makes this hexose unfit as a source of energy for the proteus and cholera microbes.⁷ Many bacteria are more versatile in this respect.⁶

The question presents itself: What part of the hexose molecule excites the greater influence in determining fermentability, the terminal group or the inter-terminal arrangement of H and OH groups?

A series of sugar derivatives was prepared by one of us (Robert Bly), including the following—gluconic and saccharic acids from d-glucose; mannonic lactone and mannosaccharic lactone from mannose; and galactonic acid from galactose. These acids in addition to the alcohols sorbitol, dulcitol, and mannitol provided a considerable, but not complete, series of hexose sugars and derivatives which differ merely in the character of the terminal groups. The interterminal

⁸ Fernbach and Schoen: Ann. d. l'Inst. Pasteur, 1914, 28, p. 692.

⁹ Duclaux: Ann. Inst. Nat. Agri., 1887, 10, p. 39.

arrangements are unchanged.¹⁰ The inter-relations of the various derivatives are indicated in the following diagram:

D-GLUCOSE	D-LAEVULOSE	D-MANNOSE	D-GALACTOSE
1. $\begin{array}{c} \text{H} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$	$\begin{array}{c} \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{C} = \text{O} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$	$\begin{array}{c} \text{H} - \text{C} = \text{O} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$	$\begin{array}{c} \text{H} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$
GLUCONIC ACID		MANNONIC ACID [Lactone]	GALACTONIC ACID
1. $\begin{array}{c} \text{HO} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$		$\begin{array}{c} \text{C} = \text{O} \\ \quad \diagup \\ \text{HO} - \text{C} - \text{H} \quad \text{O} \\ \quad \diagup \\ \text{HO} - \text{C} - \text{H} \quad \\ \quad \diagup \\ \text{H} - \text{C} - \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$	$\begin{array}{c} \text{HO} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$
SACCHARIC ACID		MANNO SACCHARIC ACID [Lactone]	MUCIC ACID
1. $\begin{array}{c} \text{HO} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} = \text{O} \end{array}$		$\begin{array}{c} \text{C} = \text{O} \\ \quad \diagup \\ \text{HO} - \text{C} - \text{H} \quad \text{O} \\ \quad \diagup \\ \text{HO} - \text{C} - \text{H} \quad \\ \quad \diagup \\ \text{H} - \text{C} - \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} = \text{O} \end{array}$	$\begin{array}{c} \text{HO} - \text{C} = \text{O} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} = \text{O} \end{array}$
SORBITOL		MANNITOL	DULCITOL
1. $\begin{array}{c} \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$		$\begin{array}{c} \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$	$\begin{array}{c} \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{HO} - \text{C} - \text{H} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} - \text{C} - \text{OH} \\ \\ \text{H} \end{array}$

¹⁰ Excepting, of course, the lactone tie between the alpha and gamma carbon atoms in the mannonic and mannosaccharic acid lactones.

The most striking result elicited from this comparative study of hexose sugars and their derivatives is the unexceptional ability of all the microbes to ferment glucose. Galactose also is utilizable for energy by a majority of the organisms described in this list.¹¹ Fructose (levulose) and mannose do not seem to fit the spacial relations of the protoplasm of the proteus group, and at least some strains of the cholera vibrio fail to utilize mannose. These observations indicate quite clearly that a common enol formation⁵ is not an absolute prerequisite to fermentation; otherwise the members of the group that

TABLE 1
REACTIONS OF ORGANISMS STUDIED IN VARIOUS MEDIUMS

	d-Glucose	Gluconic Acid	Saccharic Acid	Sorbitol	Fructose	Mannose	Mannonic Acid	Manno-Saccharic Acid	Mannitol	Galactose	Galactonic Acid	Mucic Acid	Dulcitol	
<i>Staphyl. aureus</i>	+	—	—	±	+	+	—	—	±	±	—	—	—	6 strains
<i>Mic. tetragenus</i>	+	—	—	—	+	—	—	—	—	+	—	—	—	2 strains
<i>Mic. zymogenes</i>	+	+	+	+	+	+	+	—	+	+	+	—	—	
<i>Mic. ovalis</i>	+	+	+	+	+	+	+	—	+	+	+	—	—	3 strains
<i>Str. pyogenes</i>	+	+	+	+	+	+	+	—	+	+	+	—	—	4 strains. See, Kendall, Day, Walker and Ryan ⁶
<i>Pneumococcus</i>	+	+	+	+	+	+	+	—	+	+	+	—	—	Types I - IV, in- clusive
<i>B. dysenteriae</i> , Shiga...	+	+	+	—	+	+	—	—	—	+	+	—	—	8 strains
<i>B. dysenteriae</i> , Flexner.	+	+	+	+	+	+	—	—	+	+	+	—	—	
<i>B. dysenteriae</i> , Somme..	+	+	+	—	+	+	+	—	+	+	+	—	—	3 strains
<i>B. typhosus</i>	+	+	+	+	+	+	+	—	+	+	+	—	—	2 strains
<i>B. paratyphosus</i> , alpha	g	g	g	g	g	g	+	—	g	g	g	—	—	See footnote 13 for dulcitol
<i>B. paratyphosus</i> , beta..	g	g	g	g	g	g	—	—	g	g	g	—	—	3 strains
<i>B. coli</i>	g	g	g	g	g	g	+	—	g	g	g	—	—	
<i>B. proteus</i>	g	g	g	—	g	—	—	—	g	g	g	—	—	5 strains
<i>B. mucosus capsulatus</i> ..	g	g	g	g	g	g	—	—	g	g	g	—	—	6 strains
<i>Vibrio cholerae</i>	+	+	+	—	+	±	±	—	+	+	+	—	g	4 strains

+ = acid reaction; g = gas and acid; — = no fermentation, reaction becomes gradually alkaline; ± = majority but not all strains give an acid reaction; ± = majority but not all strains fail to ferment.

possess a common enol—d-glucose, d-mannose and d-fructose—would theoretically be mutually convertible and mutually fermentable. Furthermore, gluconic and galactonic acids, which cannot readily undergo enolization, are fermentable by members of the proteus group. Here again enolization can be ruled out with considerable definiteness as a prerequisite to fermentation. On the other hand, mannitol is on the whole somewhat more fermentable than sorbitol, although the disproportion in the group studied is not great. The Flexner and Somme

¹¹ The list is by no means complete; several microbes not mentioned herein, for example, the vibrio of Finkler and Prior, fail to utilize galactose.

strains of the dysentery group utilize mannitol without difficulty, but the latter fails to find in sorbitol the requisite totality of configuration necessary for intracellular decomposition for energy. It is not the configuration of the asymmetric carbon atom groups presumably that prevents the fermentation of mannitol, because mannose, which possesses precisely the same configuration except for the aldehyde group, is readily decomposed. It will be remembered that the Shiga dysentery strains ferment glycerol readily, whereas the Flexner and Somme strains thus far studied do not.

In general, it may be said that the aldose and ketose hexose sugars of this series are on the whole readily fermented by a great majority of the strains.

The corresponding alcohols, mannitol, sorbitol and dulcitol, offer increasing difficulties to microbic decomposition in the order given, with a disproportionate refractoriness manifested by dulcitol. Indeed, dulcitol is attacked by comparatively few microbes, which are on the whole unusually active in their fermentation reactions.¹² The mucosus capsulatus group is the most conspicuous of these. It should be mentioned in passing that this group, and *Bacillus paratyphosus* beta, ferment glycol, a diatomic alcohol. This substance is even more unavailable than dulcitol for energy to most bacteria.

Gluconic acid, in which the aldehyde group of d-glucose is oxidized to a carboxyl group, is usually as fermentable as the aldehyde itself. The noteworthy exception is the *Staphylococcus* group, including *Micrococcus tetragenus*. Galactonic and mannonic acid lactone are about as readily decomposed by microbic activity as gluconic acid. There is no known instance of the acid being fermentable, however, when the aldehyde is not fermented.

Saccharic acid, in which the terminal carbon atoms of glucose are oxidized to carboxyl groups, seems to be nearly, if not quite, as readily utilized as gluconic acid, and even glucose itself. This is somewhat unexpected. Mannosaccharic acid lactone and mucic acid, on the contrary, are very refractory to microbic fermentation. It should be stated that a few strains of *Bacillus mucosus capsulatus*, freshly isolated and active in their fermentation reactions, do utilize mannosaccharic acid lactone. Thus far these are the only aerobic bacteria encountered which can derive energy from this substance.

¹² Some strains of the paratyphoid group, however, ferment dulcitol slowly. Usually these strains are of the enteritidis-suipestifer group, see Jordan, *Jour. Infect. Diseases*, 1917, 20, p. 457.

The evidence deduced from this study throws little additional light on the relation between the configuration of certain hexose sugars and their derivatives and their utilizability by the more common laboratory strains of bacteria which are parasitic on or pathogenic for man. Certain group tendencies, as for example the inability of most staphylococci to ferment any except the aldose-ketose sugars, and the failure of the proteus group to utilize fructose and mannose configurations, stand out quite clearly. On the other hand, with the exception of mannosaccharic acid and mucic acid, the mono and dicarboxyl acids derived from these hexoses are nearly as adaptable to the energy requirements of the microbes as the sugars themselves. Dulcitol stands out conspicuously among the hexatomic alcohol derivatives, because of its noteworthy refractoriness to fermentation.

For purposes of diagnosis and bacterial classification, it would seem a waste of time to depart from the custom of the past, and introduce these various acid derivatives of the hexoses as supplementary means of identification. In this regard the negative evidence herewith presented may be considered informing, but not creative.

The major premise, namely, the influence of the terminal group in the hexose sugars and their derivatives on microbial utilization has received some statistical support, but intimate knowledge of the underlying principle of the relation between carbohydrate configuration and protoplasmic decomposition has not been materially advanced.

ETIOLOGY OF SPONTANEOUS ULCER OF STOMACH IN DOMESTIC ANIMALS

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After demonstrating that ulcer of the stomach in man appears to be due to a localized infection, and that the streptococcus isolated from the ulcers and from foci of infection in patients with ulcer localized electively in the wall of the stomach, producing ulcer,¹ I believed that a similar study might throw light on the etiology of ulcer in animals, and at the same time test the applicability of the methods evolved. Through the coöperation of Armour and Company, Chicago, opportunity was afforded to study ulcer of the stomach in the calf, cow, and sheep. The ulcers in the dogs were found during the examination of a large number of dogs which were killed by gas at the pound. The method of study was similar to that in the study of ulcer in man. The stomach was opened and the contents removed so as to avoid gross contamination from the outside. The ulcer-bearing or hemorrhagic, infiltrated areas, and in some instances normal mucous membrane, were washed in running water and then excised with sterile instruments. Some of the excised tissues were immediately placed in sterile containers, properly iced, for cultures, and others were placed in 10% formaldehyd for microscopic study. The factor of surface contamination was eliminated so far as possible by searing the ulcerated area, by excising pieces of the submucosa and peritoneal layers free from the ulcerated surface in the ulcers that were markedly indurated, and by repeatedly washing, in large amounts of sodium chlorid solution, the ulcers that were slightly thickened. These tissues were then mascerated with mortar and pestle in sterile air chambers and suspended in salt solution. Cultures were made by inoculating varying amounts of the emulsified tissue into a series of tubes or bottles containing tall columns of glucose and ascites-glucose broth and melted agar, and into plain and glucose-blood-agar plates. By using a series of flasks of broth, by inoculating varying amounts of the emulsion, and by eliminating contaminating organisms so far as possible, pure growths were often obtained in sufficient amount for the injection of animals with the primary culture. The shake cultures in glucose-agar, also inoculated with varying amounts of the

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¹ Jour. Infect. Dis., 1916, 19, p. 333.

emulsion, afforded conditions favorable for the maintenance of the specific infecting power of the bacteria and for obtaining pure cultures in broth for animal inoculation, especially when the primary culture in broth contained gas-producing and other saprophytic bacteria in spite of the precautions taken.

Rabbits and dogs were used as test animals. Injections were given intravenously. The dose varied within wide limits, depending on the purpose. Usually the growth of from 2 to 8 c c. of the broth for each kilogram of body weight, suspended in sodium chlorid solution, was injected rather rapidly into the marginal ear vein in rabbits, and into a leg vein in dogs. The number of animals injected was determined in the main by the character of the ulcer and by the results of the cultures. The animals were fed greens, hay, oats, and water, and were observed daily. Those that survived the injection were chloroformed for examination, usually in from 2 to 4 days, sometimes in 7 days, after injection. Search for lesions was made in a bright light and the results recorded.

These experiments were performed at the John McCormick Institute for Infectious Diseases, Chicago, from January to May, 1915. Publication of the work was postponed in order to include similar experiments with certain of the cultures after long residence in artificial mediums.

In testing the viability and infecting power of old cultures from various diseases recently, shake cultures of several ulcer strains in ascites-glucose-agar, layered with oil, were found to be viable and to have retained their affinity for the stomach after 7½ years. This result, and the fact that the cause of ulcer of the stomach in dogs and other animals is still obscure, led to detailed analysis of the results obtained in the experiments performed in 1915 and in those performed recently.

RESULTS

The incidence of ulcer in the calf, cow, and sheep was about 1 to 60, that in the dog, 1 to 40. In discussing the question with the men at the abattoir whose duty it was to open the stomachs of calves, cows, and sheep, I learned that the incidence of ulcer in the cow and the sheep was highest each year during the latter part of the winter and early spring months, and that in their opinion the ulceration was the result of eating dry, coarse foods during these months, since the condition disappeared almost entirely each year during the summer and fall months when the animals were in pasture. The finding of large healed scars in the mucous membrane indicates that this factor in the diet may play a part.

The character of the ulcers varied greatly in different animals in the same species, and in the animals in the different species. Those in the first 3 stomachs of the calf, cow, and sheep were usually large, irregular in outline (fig. 1), superficial, and associated with marked thickening and infiltration of the 3 coats, and with a variable amount of fibrinous and fibrous peritonitis. The evidence of infection was usually striking. The ulcers in the hog and the dog, and those in the acid-

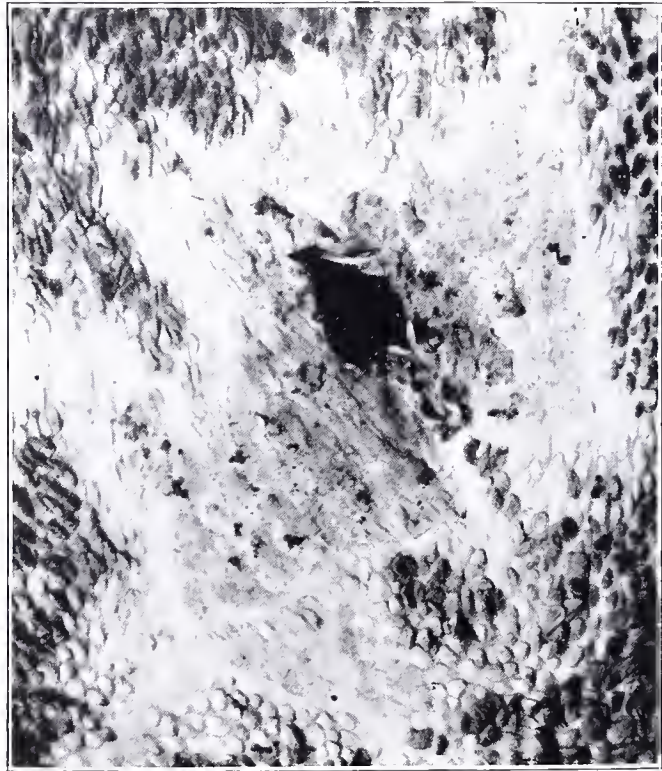


Fig. 1.—Large irregular superficial ulcer of the third stomach (omasum) of a sheep.

secreting stomach (abomasum) of the ruminant animals were smaller, more sharply localized, associated with relatively little infiltration and rarely with adhesions. They often resembled the so-called peptic ulcer in man (fig. 2). Acute and chronic ulcers and healed scars were found in the same animal. Large and small localized areas of hemorrhages and infiltration without ulceration, with beginning ulceration, and with marked ulceration were also found. In no instance did I find ulcers resembling the chronic deep crater-like ulcer so common in man.

In the investigation of the cause of ulcer in man, foci of infection, such as are commonly found in teeth and tonsils, were found to harbor streptococci that tended to produce ulcer of the stomach, quite as did the strains isolated from the ulcers.¹ When it became apparent that the streptococcus was the predominating organism in the ulcers of animals and that it produced ulcer on intravenous injection in rabbits and dogs, the factor of focal infection was studied. No evidence of infection of the teeth of calf, cow, sheep or hog was found in the

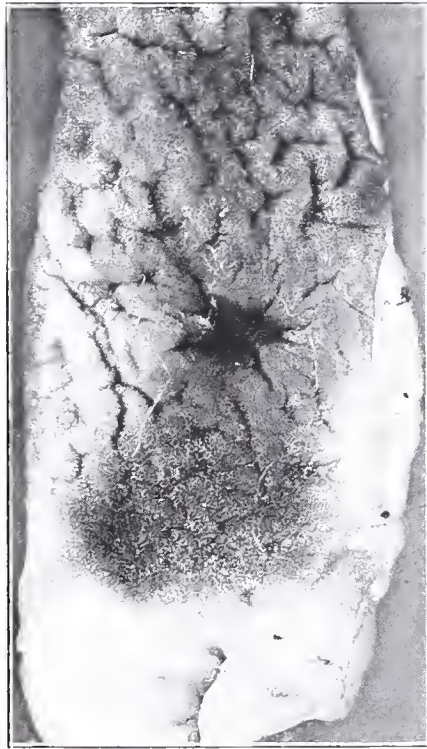


Fig. 2.—Spontaneous deep ulcer in the stomach of a dog, from which a streptococcus having elective affinity for the stomach was isolated ($\times 3$).

animals that had ulcer. In a number of the dogs which had ulcer, marked infection, resembling pyorrhea, was found around the gums, always associated with infection in the tonsils. One or both tonsils of a number of sheep and cows revealed marked infection. In 2 instances this appeared to be due to trauma, because small slivers of corn stalk were embedded in well formed abscesses. Streptococci resembling those from the ulcers in cultural characteristics and infecting power were isolated from infected tonsils in 3 instances each in cow and dog.

The findings in sections of the tonsils in these animals were interesting. The lymphoid follicles for the most part were normal. In some, leukocytic infiltration and small abscesses were found. Most of the crypts were empty and lined with a thick layer of squamous epithelium, but a number were distended with pus. In these the epithelium was desquamated in areas, and the surrounding interstitial tissue and lymphoid follicles were infiltrated with leukocytes (fig. 3). Sections stained by Gram revealed a moderate number of diplococci, usually within leuko-

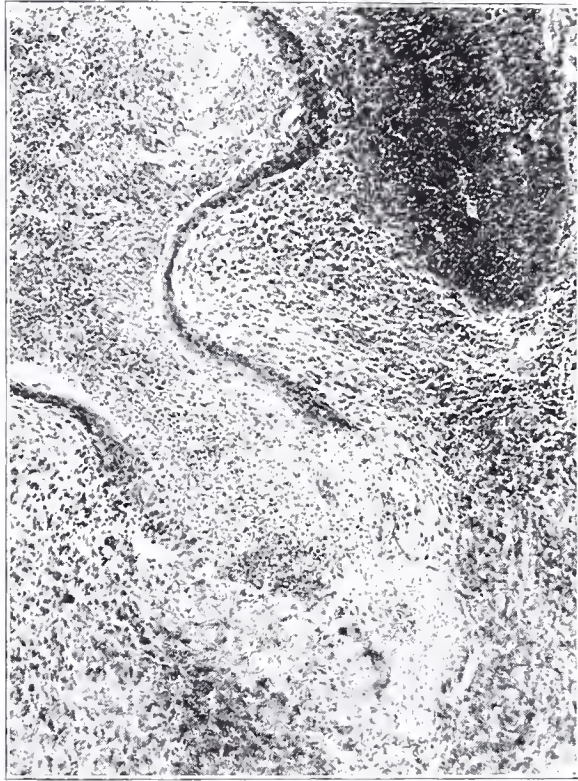


Fig. 3.—Abscess in the tonsil of a cow having ulcer, from which a streptococcus which produced ulcer in rabbits and dogs was isolated. Hematoxylin and eosin ($\times 100$).

cytes, in the pus filling the crypts, and an occasional diplococcus in the areas of hemorrhage and infiltration surrounding the crypt lining. Three of the dogs with ulcer of the stomach also had cholecystitis, acute in 1, and chronic in 2. The gallbladder of 1 of the latter contained small gallstones embedded in thick, black bile.

Cultures were made of material from 70 animals, of ulcers from 61, of areas of hemorrhage and infiltration without ulceration from 9, and

of normal mucous membrane and healed scars remote from ulcers as controls from 14. Of the 61 ulcers from which cultures were taken, streptococci in pure culture or in predominating numbers were isolated in 57, and no bacteria or only a few saprophytes in 4. The 9 areas of hemorrhagic infiltration, all found in calves, yielded the streptococcus. What was regarded as normal mucous membrane in 8 instances proved sterile in 5, whereas in 3, a few colonies of streptococci and other bacteria developed in glucose-agar shake cultures. The cultures of the 6 healed scars yielded a few colonies of streptococci in 1; the others were sterile or contained only a few saprophytic bacteria. The results of the cultures according to species were as follows:

Ulcers and hemorrhagic areas from 13 calves yielded the streptococcus in every instance, ulcers from 14 cows in 11, ulcers from 8 dogs

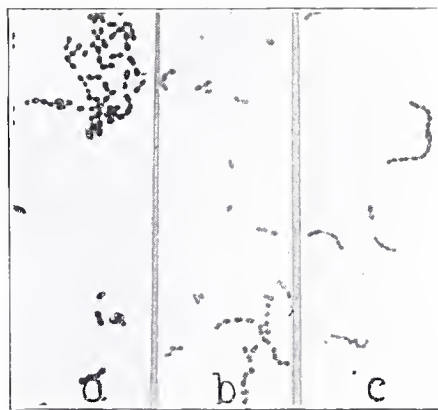


Fig. 4.—Smears from ascites-glucose-broth cultures of streptococci from ulcer, *a*, in the sheep, *b*, in the cow, *c*, in the dog, which produced ulcer of the stomach on intravenous injection into rabbits and dogs. Gram ($\times 1000$).

in 7, ulcers from 5 hogs in 4, and ulcers from 30 sheep in all. The number of colonies of streptococci that developed varied greatly. In some instances countless numbers developed in the shake cultures, in others the colonies were few, and sometimes absent, when the broth cultures yielded a pure growth. The strains from the calf, cow, and sheep produced rather large, flat, sometimes umbilicated colonies surrounded by a marked green zone on blood-agar, and had a marked and wide range of fermentative power over sugars, producing marked acidity in glucose, maltose, lactose, mannite, salicin, inulin, and saccharose. They grew in clumps and in rather long chains of diplococci in broth, producing diffuse turbidity (fig. 4*a* and *b*). Two of the

5 strains from the ulcers in hogs and 2 of the 8 in the dogs produced hemolysis on blood-agar; the others produced small dry colonies surrounded by a narrow green zone on blood-agar, and diffuse turbidity in broth; smears showed clumps and short chains of diplococci (fig. 4c).

In 23 instances, cultures were made of the peritoneal or muscular coats of markedly infiltrated ulcers and of excised ulcers after searing the surface, without coming in contact with the ulceration. Pure cultures of the streptococcus, usually in large numbers, were obtained in 18, and a predominating number of streptococci, with few other bacteria, usually *Bacillus coli*, in 5.

Sections of hemorrhagic areas and of ulcers in the nonacid-secreting stomach of the calf, cow, and sheep revealed marked diffuse leuko-

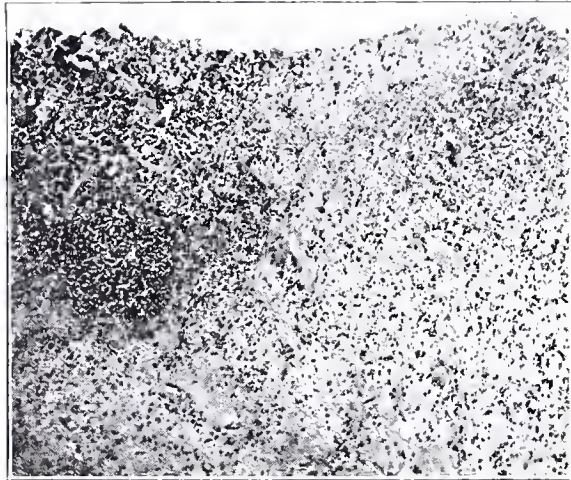


Fig. 5.—Section of ulcer shown in Figure 1. Note the marked leukocytic infiltration. Hematoxylin and eosin ($\times 60$).

cytic infiltration, in many instances extending through the submucosa, and muscular and peritoneal coats (fig. 5). The cells of the mucous membrane were necrotic for some distance beyond the ulcerated area, especially where leukocytic infiltration in the submucosa was marked. Thrombosis was not found in any of these ulcers. The demonstration of diplococci, sometimes in short chains, was readily accomplished on the ulcerated surface beneath the necrotic epithelium along the margin of the ulcer, and sometimes in the peritoneal coat of acute ulcers. It was more difficult in chronic ulcers. Other bacteria, chiefly small gram-negative bacilli, were often found in small numbers on the free surface of the ulcers, but not in the infiltrated tissues remote from the ulcerated areas.

The microscopic findings in the areas of hemorrhage and ulceration in the mucous membrane of the acid-secreting stomach of these species, and in the hog and the dog, were similar to those of the peptic ulcer in man. The ulcers were small, and the base and margins were usually clean and free from necrotic tissue. The hemorrhage and leukocytic infiltrations were sharply circumscribed, relatively slight, and usually limited to the submucosa, but in some instances extended up between the gastric glands to the free surface of beginning ulcerations, and only rarely into the muscular and peritoneal coats. Thrombosis of small vessels, always in the submucosa, was found in 4 instances, all in relatively acute ulcers. The demonstration of bacteria in these ulcers was more difficult than in those of the nonacid-secreting stomachs, but

TABLE 1
INCIDENCE OF FOCAL LESIONS IN VARIOUS ORGANS FOLLOWING INTRAVENOUS INJECTION OF STREPTOCOCCI FROM ULCER OF THE STOMACH IN ANIMALS

Species	Strains Injected	Animals Injected	Eyes	Muscles	Joints	Intestines	Appendix	Stomach		Duodenal Hemorrhage	Total Number of Animals with Lesions in Stomach or Duodenum	Gallbladder	Pancreas	Kidneys	Lungs	Pericardium	Myocardium	Endocardium
								Hemorrhage	Ulcer									
Sheep.....	11	20	0	0	5	3	0	15	16	0	17	9	2	0	0	2	1	2
Cow.....	7	23	3	5	6	0	1	14	18	6	19	6	0	2	1	0	0	0
Calf.....	12	0	2	0	0	3	3	11	8	4	12	1	0	0	2	0	2	1
Dog.....	5	23	0	2	7	1	0	16	11	0	19	8	0	1	0	0	2	0
Total.....	30	78	3	9	18	7	4	56	53	10	67	24	2	3	3	2	6	3
Percentage of animals with lesions in.....			4	12	23	9	5	71	68	13	86	31	3	4	4	3	8	4

was accomplished in all of the acute ulcers. Bacilli and streptococci were often found on the ulcerating surface, and diplococci only in the depths of the surrounding infiltration. In chronic ulcers, bacteria were not always demonstrable.

Experiments were not conducted with the strains of streptococci from the hog. The results following intravenous injection of streptococci from the sheep, cow, calf, and dog are summarized in table 1.

Fifty-five rabbits and 23 dogs were injected. The virulency of the strains was of a low order. Despite the injection of large numbers of the streptococci, only 12 of the rabbits and 4 of the dogs (20%) died. The rest appeared well when chloroformed for examination. The incidence of lesions in various organs in the 2 species was similar.

A high incidence of lesions in the stomach and duodenum occurred consistently following injection of the streptococcus in the 4 species studied. The affinity for the stomach was greater than for the duodenum, localized hemorrhages of the stomach occurring in 71% of the animals, ulcer in 68%, hemorrhages of the duodenum in only 13%, and ulcer of the duodenum not at all. Noteworthy lesions of the stomach or duodenum occurred in 67 (86%) of all animals injected. The relatively high incidence of lesions in the gallbladder (31%), which

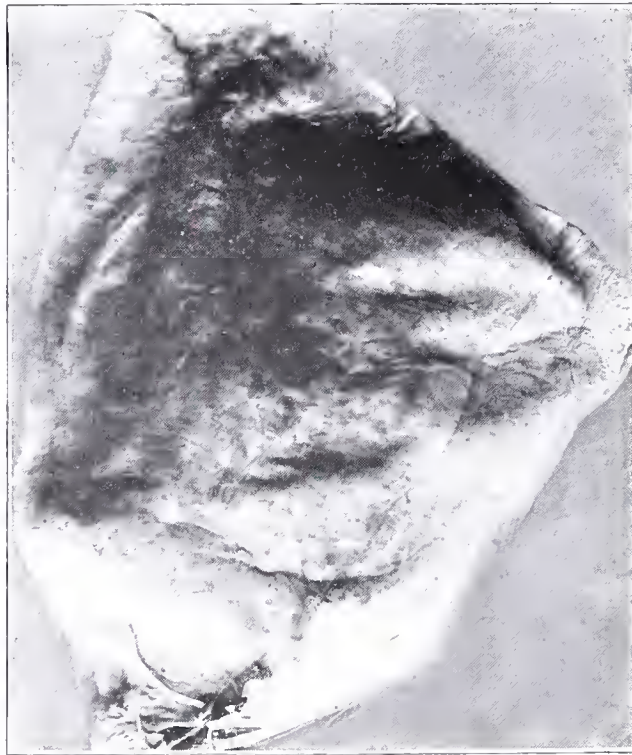


Fig. 6.—Marked hemorrhagic infiltration beneath the peritoneal coat in the fundus of the stomach of a dog injected with the streptococcus isolated from the ulcer of the sheep shown in Figure 1.

was more than twice as high in the dogs as in the rabbits, is in keeping with the fact that cholecystitis and ulcer occurred in the same animal in 3 of the 8 dogs with spontaneous ulcer, with the fact that cholecystitis and ulcer in man are often present in the same patient, and with the results of the experiments with ulcer strains from man.

The incidence of lesions in other organs corresponded closely to that obtained in other studies on the localization of streptococci. The

joints, as with other than ulcer strains, showed relatively high susceptibility (23%).² In no series of animals injected with streptococci from sources other than ulcer in man² and animals³ has such a high percentage of localization in the stomach been obtained.

The findings in the experimental lesions in the stomach of rabbits and dogs, like those in the spontaneous lesions, indicated that ulcera-

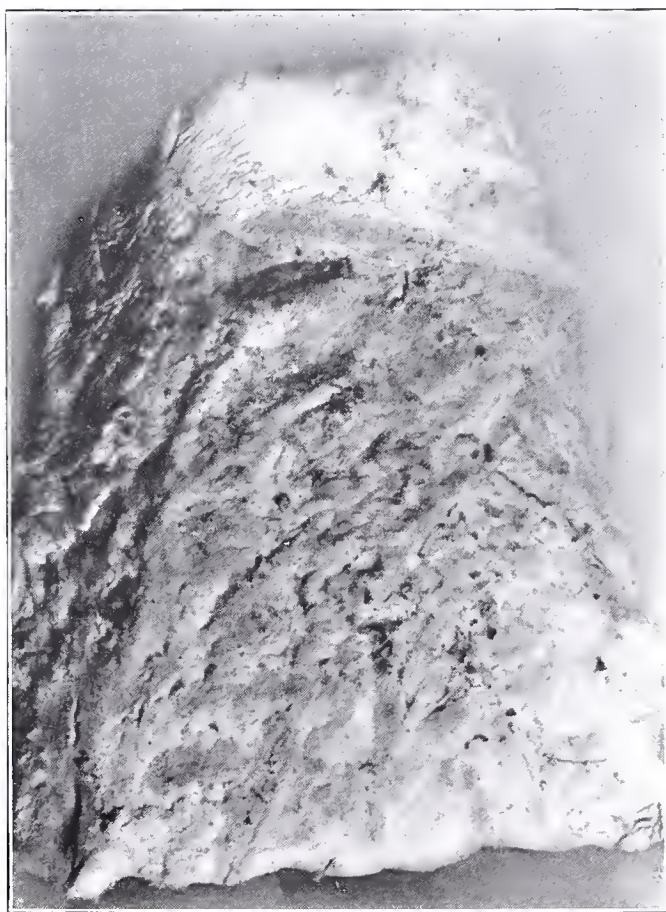


Fig. 7.—Localized hemorrhages, superficial erosions, and localized ulcerations of the stomach of a dog injected with the streptococcus from a spontaneous ulcer in a dog.

tion was often due to areas of localized infection in the wall of the stomach. The areas of hemorrhage were often associated with edema and infiltration. The lesions following injection of strains from the

² Jour. Am. Med. Assn., 1915, 65, p. 1687.

³ Arch. Int. Med., in press.

large spreading ulcers of the nonacid-secreting stomach of the cow and sheep, with marked infiltration of, and adhesions to, the peritoneal coat, were often diffuse, and in some instances revealed large areas containing coalescing hemorrhages in and beneath the peritoneal coat (fig. 6). Those following injection of the streptococcus from the ulcer in the acid-secreting stomach in the calf, cow, sheep, and dog, as well as those from the infected tonsils of the cow and the dog having ulcer, were more sharply localized (figs. 7 and 8).

Cultures from the blood were made in 51 of the injected animals. Of these, only 13 yielded the streptococcus. Cultures of experimentally



Fig. 8.—Hemorrhagic, edematous, infiltrated areas with beginning ulceration in the pyloric end of the stomach of a rabbit injected with the streptococcus isolated from the infected tonsils of a dog that had ulcer of the stomach.

produced ulcers were made in 29 animals. Of these, 23 (79%) yielded the streptococcus injected, which in some instances on reinjection produced hemorrhage or ulcer of the stomach. The streptococcus was demonstrated in the experimentally produced lesions in the stomach in one or more instances following injection of strains from each of the species studied.

ILLUSTRATIVE EXPERIMENTS

The wall of the stomach of one of the sheep contained a number of large, irregular, ulcerated areas, with intervening portions of fibrous tissue (fig. 1).

The wall in this area was markedly thickened and the peritoneum was adherent to the omentum. Cultures were made separately of the ulcer and peritoneal coat; the former yielded 120, the latter 14 colonies of streptococci in pure culture in ascites-glucose-agar. The primary culture of the ulcer strain in ascites-glucose-broth (fig. 4a) was injected into 2 dogs, and 1 rabbit. One of the dogs died in 24 hours. The stomach showed large areas containing numerous punctate confluent hemorrhages beneath the peritoneum (fig. 6), and in the mucous membrane of the pyloric end. Cultures from the areas of hemorrhage and from the blood yielded a pure growth of the streptococcus, whereas control cultures of joint fluid and bile were sterile. The other dog was well at the end of 6 days when chloroformed. It had 2 healing ulcers in the pyloric end of the stomach, and one in the cardiac end, and a fading hemorrhage in the fundus of the gallbladder. The rabbit was chloroformed at the end of 48 hours. Necropsy revealed a large hemorrhage and an infiltrated area in the pyloric end of the stomach, and a few lesions in the muscles and myocardium. The 1 rabbit injected with the strain from the first dog mentioned died in 3 days. Necropsy revealed marked edema and infiltration extending through to the peritoneum of the pyloric end of the stomach, and marked hemorrhage and ulceration of the mucous membrane in the cardiac end.

A series of experiments were performed with one of the strains isolated from an ulcer and from the tonsils in a cow. The stomach of this cow contained an infected indurated area, approximately 7 cm. in diameter, in the center of which a large ulcer was found. The wall of the stomach at this point was fully 3 times the normal thickness. The surface of the ulcer was seared and a part excised, emulsified, and cultures made. The glucose-agar cultures yielded a large number of colonies of streptococci, and the ascites-glucose-broth an almost pure growth of the streptococcus and a few staphylococci. The tonsils were large and hyperemic, and contained numerous small abscesses surrounded by hyperemia and hemorrhages. Smears from the pus showed large gram-positive bacilli resembling *Bacillus welchii*, and streptococci. The cultures of the pus on glucose-agar plates contained approximately 500 colonies of streptococci and a few colonies of staphylococci, and in ascites-glucose broth a pure growth of the streptococcus resembling the one from the ulcer was obtained.

The culture from the ulcer was injected into 2 dogs and 4 rabbits. The dogs were chloroformed 48 hours later. Both had hemorrhages and ulcers in the stomach and no lesions elsewhere. Two of the rabbits were chloroformed in 24 hours, and 2 in 48 hours. The first 2 had subperitoneal and submucous hemorrhages along the lesser curvature of the stomach, and a few hemorrhages in muscles. The stomach of one of the latter contained an ulcer 2 cm. from the cardiac end in the center of a hyperemic infiltrated area, 1 cm. in diameter. In the other an ulcer 0.5 cm. in diameter had developed along the lesser curvature; no lesions were found elsewhere. Cultures were made of the blood and ulcers from 5 of the animals. The blood was sterile in 4, whereas the cultures from the ulcers yielded streptococci in 2 of the rabbits and in 1 of the dogs.

The streptococcus from the tonsil was injected into 2 dogs and 2 rabbits. All were seemingly well 48 hours later, when they were chloroformed. Both dogs had hemorrhages and ulcer in the stomach, and 1 also had hemorrhages in the wall of the gallbladder. Cultures from the blood were sterile in both; those from the ulcers revealed the streptococcus in 1, while in the other only saprophytic bacteria were found. One of the rabbits developed a large area of hemorrhagic infiltration, with beginning ulceration, in the pyloric one-third; the other developed a similar area near the cardiac orifice. The latter also

had a lesion resembling herpes in the conjunctiva at the inner angle of the right eye. Cultures from the blood and the clear joint fluid in both remained sterile.

The findings and results of experiments in one of the dogs were especially interesting. Necropsy revealed a markedly distended stomach, with acid contents and gas rich in carbon dioxide. The mucous membrane was hyperemic throughout and contained numerous circumscribed areas of infiltration and superficial erosions, and several deep, round ulcers with a hemorrhagic area along the lesser curvature. A scar, evidently of a healed ulcer, was found in the duodenum. Both lobes of the thyroid were enlarged. The tonsils were hyperemic, and on pressure, pus exuded from the single deep crypt. An area of fibrous thickening 1.5 by 2.5 cm. was found in the fundus of the gallbladder. The other viscera were free from changes. The emulsion of one of the ulcers yielded, on blood-agar plates and in glucose-agar shake cultures, a large number of colonies of a short-chained streptococcus and a few gram-positive and gram-negative bacilli, and a preponderance of the streptococci in an 18-hour broth culture. The shake cultures of emulsion of the tonsils, after searing the surface, yielded fully 5,000 colonies of the same streptococcus, and a few colonies of staphylococci and bacilli.

Three dogs and 2 rabbits were injected with the streptococcus from the ulcer in the second culture (fig. 4c). The 5 animals appeared well after 48 hours, when they were chloroformed. The 3 dogs had lesions of the stomach resembling those shown in Figure 7, and one also had marked, the other slight localized, hemorrhages in the gallbladder. The stomach of one of the rabbits contained marked hemorrhages, infiltrations, and ulcers, especially near the pyloric ring, and embolic areas in the gallbladder; in the other rabbit, a group of small hemorrhages in the duodenum, embolic lesions in the medulla of the kidney, and mild arthritis were found. The streptococcus isolated from the ulcer of the stomach shown in Figure 7 was injected into 2 rabbits and 1 dog. One of the rabbits appeared well 24 hours later, when it was chloroformed, and was free from lesions. The other died in 24 hours, and necropsy revealed multiple ulcerations in the stomach. The dog appeared well 4 days later, when chloroformed. A few fading hemorrhages of the mucous membrane of the stomach, and marked cholecystitis were found.

The streptococcus from the tonsil was injected intravenously into 1 dog and 2 rabbits. All the animals developed marked hemorrhages and ulceration of the stomach (fig. 8). The dog also had marked hemorrhage and edema of the gallbladder, and one of the rabbits had hemorrhages in the appendix.

RECENT EXPERIMENTS

The primary culture in ascites-glucose-broth of the streptococcus isolated from one of the ulcers in a sheep was injected intravenously into 1 rabbit May 1, 1915. The animal developed localized hemorrhages, with beginning ulceration of the mucous membrane of the pylorus, and no lesions elsewhere. One of the shake cultures in ascites-glucose-agar of the emulsion of the ulcer in this sheep, made April 30, 1915, yielded approximately 400 colonies of the streptococcus, which on plating in blood-agar produced rather large, flat colonies surrounded by a marked greenish zone. This tube was layered with paraffin oil and kept in the dark at room temperature until Oct. 28, 1922 (7½ years). At this time, subcultures were made and a pure growth of the streptococcus was isolated. It still produced a greenish zone on blood-agar plates, but this was slight.

The second culture in glucose-brain-broth was injected intravenously into 4 rabbits in doses ranging from 5 c.c. of the undiluted culture to 5 c.c. of the culture diluted 1:100 with sodium chlorid solution. All appeared well 24 hours later, when chloroformed. One, injected with a 1:10 dilution, was free from lesions; two showed small areas of hyperemia, hemorrhages, and infiltration in the mucous membrane of the stomach, associated in one with ulceration, and one a small hemorrhage in the duodenum and a few linear streaks in the medulla of the kidney and in muscles.

Six other rabbits and 2 dogs were injected with this strain in the 10th, 11th and 23d rapidly made subcultures, 3 rabbits and 1 dog before, and 3 rabbits and 1 dog after 1 animal passage. Both dogs and all but 1 rabbit developed lesions of the stomach or duodenum. Cultures from the blood of the dogs

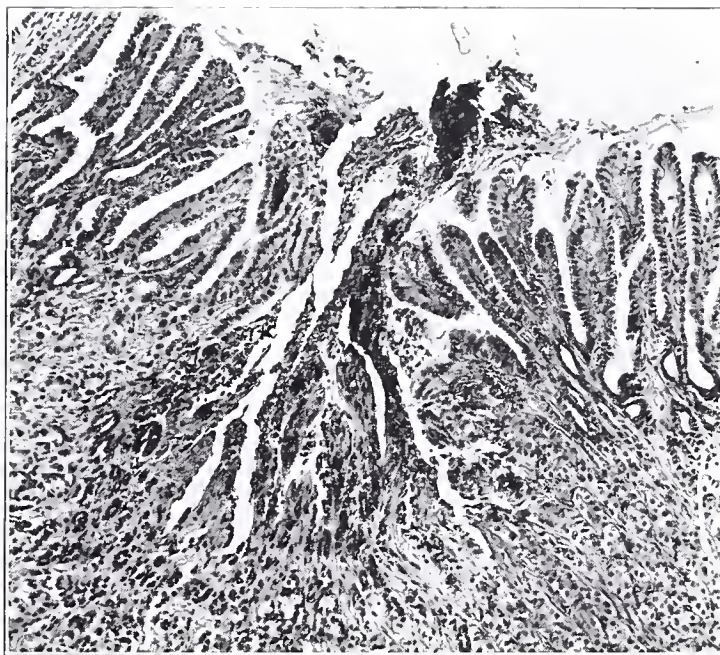


Fig. 9.—Hemorrhage, and leukocytic infiltration with beginning ulceration of the mucous membrane of the stomach of a rabbit 2 days after intravenous injection with a fresh culture of a streptococcus from an ulcer in a sheep after it was kept in latent life for $7\frac{1}{2}$ years. Hematoxylin and eosin ($\times 100$).

and from 8 of the 10 rabbits injected remained sterile; from the other 2 the streptococcus was isolated. The streptococcus was isolated from the hemorrhagic or ulcerated mucous membrane in 3 of the 5 rabbits from which cultures were taken. The gross lesions, while not so extensive following injection of this strain so long after isolation, were similar to those following injection of freshly isolated strains, and the incidence of specific localization was as high. The mortality rate was lower, and concomitantly the lesions in organs other than the stomach were less common and less marked. The microscopic changes in the lesions in the mucous membrane of the stomach were essentially the same. Hemorrhage and leukocytic infiltration were minimal (fig. 9). The

streptococcus was readily demonstrable in sloughing tissue (fig. 10), but more difficult to find in the tissues adjacent to the ulcerating surface, including areas of leukocytic infiltration.

The filtrate of a mixture of the heated broth cultures from the 2d to the 21st rapidly made subcultures, and the corresponding heat-killed bacteria (60 C. for 1 hour) were injected into 2 rabbits each. The dose was 10 c.c. of the filtrate for each kilogram, and the growth from 30 c.c. of the glucose-brain-broth culture of the dead bacteria. The 4 rabbits developed hemorrhages of the mucous membrane of the stomach; in 2, this was associated with marked ulceration. The microscopic changes in these lesions were similar to those following injection of the corresponding live cultures. Bacteria were not demonstrable in lesions that developed following injection of the filtrate, but they were found in the lesions following injection of the dead bacteria. Cultures from the hemorrhagic areas and the blood in these lesions remained sterile.

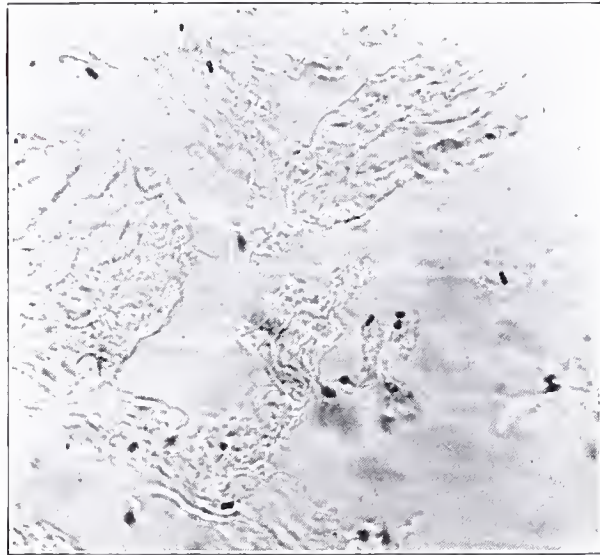


Fig. 10.—Diplococci in the sloughing tissue shown in Figure 9. Gram ($\times 1000$).

The 2 rabbits injected as controls with the corresponding dose of the uninoculated broth brought to the same degree of acidity with acetic acid had no lesions in the mucous membrane of the stomach, or elsewhere.

In order to rule out the possibility of accidental contamination, a second culture was made of the original tube, with identical results. A third culture made after the tube was exposed to diffuse sunlight for 10 days proved sterile.

One other strain from ulcer in a sheep which produced ulcer in rabbits on isolation, and which was kept in latent life for $7\frac{1}{2}$ years, proved viable, and also had retained elective localizing power for the mucous membrane of the stomach of rabbits. A third strain, while viable, had lost this property. The selective action appears to be due to a toxic substance or complex of substances demonstrable in filtrates of actively growing cultures of these strains and in the heat-killed organisms.

CONCLUSIONS

Streptococci were isolated consistently in pure cultures or in predominating numbers from a series of ulcers in the hog, calf, cow, sheep, and dog. This organism was demonstrable in the tissues, and the number was roughly proportional to the acuteness of the condition. There was an increasing incidence of pure cultures in the tissues as the distance from the source of contamination, the ulcerated surface, increased. Ulcer, hemorrhage, and infiltration of the stomach were produced in 86% of rabbits and dogs injected with the freshly isolated cultures from ulcers in the different species, often with no lesions elsewhere. Similar results were obtained with cultures of the streptococcus from the infected tonsils in the cow and dog that had ulcer. The streptococcus was demonstrated in, and isolated from, experimentally produced ulcers when other tissues were sterile, and produced ulcer on reinjection. Specific localizing powers were retained for 7½ years in 2 of 3 strains preserved in ascites-glucose-agar shake cultures, layered with oil.

Results such as these have not been obtained with streptococci of similar morphology from sources other than ulcer. Ulcer of the stomach in animals, as in man, is apparently often due to localized blood-borne infection by streptococci having selective affinity for the mucous membrane or other parts of the stomach.

INFECTIOUS ABORTION IN DOMESTIC ANIMALS

I. INFECTION OF SWINE AND RABBITS

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During the past few years, abortion in swine has been attracting more attention than formerly. Whether the disease has been overlooked or whether it actually has been increasing, is not certain. Although it has been known for 25 years that *Bacterium abortum*¹ was the cause of much of the abortion in cattle, the first recorded isolation of this organism from an aborted swine fetus was made in 1914 by Traum,² at that time of the U. S. Bureau of Animal Industry. The account of this work, included in a report on other swine diseases, was unindexed and hence escaped the attention of workers for a time. Later Good and Smith³ of Kentucky isolated *B. abortum* during an outbreak of abortion in a herd of swine. With the culture obtained, they produced abortion in one sow by feeding and in another by intravenous injection. Connaway, Durant and Neuman⁴ in Missouri reported positive complement-fixation tests with *B. abortum* in 3 of 4 herds of swine in which contagious abortion was suspected. No bacteriologic data were given. In California, Hayes and Traum,⁵ investigating abortion in 3 herds of swine, found the causative organism to be *B. abortum*. Doyle and Spray⁶ reported similar findings in Indiana. In Germany, Schlegel⁷ isolated the organism in an outbreak among swine. These reports indicate that infection of swine by *B. abortum* or by an organism closely allied to the bovine strain is geographically widely distributed.

My own work includes observations made to determine to what extent the general swine population, as observed in one of the large packing centers, was infected by *B. abortum* and also includes experi-

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¹ In the final report of the committee of the Society of American Bacteriologists on characterization and classification, *Jour. Bacteriol.*, 1920, 5, p. 221, the name, *Bacillus abortum* (Bang) was changed to *Bacterium abortum* (Bang). Therefore, this name will be used throughout the paper.

² Report of Chief of Bureau of Animal Industry, 1914, p. 86.

³ *Jour. Bacteriol.*, 1916, 1, p. 415.

⁴ *Mo. Agric. Exper. Station Bull.*, 1917, 151, p. 61.

⁵ *North Amer. Vet.*, 1920, 1, p. 58.

⁶ *Jour. Infect. Dis.*, 1920, 27, p. 165.

⁷ *Ztschr. f. Infektionskr. Haust.*, 1918, 19, p. 332; abstr. *Jour. Amer. Vet. Med. Assn.*, 1920, 58, p. 206.

ments on the infection of swine by feeding bovine cultures and on the susceptibility of rabbits to infection through the alimentary and genital tracts.

EXAMINATION OF SWINE UTERUS

A large number of gravid and nongravid uteri were examined for *B. abortum*. Specimens were obtained from one of the large abattoirs at the Union Stock Yards of Chicago. Provision was made whereby the uteri, instead of being discarded immediately after removal, were thrown into a container. They were removed one at a time for examination, which was made at the abattoir. The time between removal from the carcass and examination was rarely in excess of one hour and cultures from any material obtained were planted the same day, as soon afterward as time would permit. In opening the uterus, the surfaces to be cut were painted heavily with iodine, and when dry were seared with a hot knife blade. Scalpels were held in alcohol and wiped with sterilized cotton before use. Suspected material was taken up on sterile cotton swabs in test tubes or removed with tweezers and placed in bottles or Petri dishes, the method of collection varying with the nature of the substance wanted.

Smears made in a routine manner were stained with methylene blue, carbol fuchsin and Gram's stain. Material to be cultivated was planted at times on plates of dextrose agar and always on plates of dextrose serum agar, made by adding approximately 10% of sterile sheep serum before the medium had set. Both sets of plates were incubated for 4 days, the former, in ordinary atmosphere and the latter in an atmosphere modified by the growth of *Bacillus subtilis* according to the method of Nowak.⁸

When the ordinary glass topped Petri dishes were held in closed jars, water of condensation, collecting on both the inside and the outside of the dish, formed a continuous path for the entrance of motile organisms which frequently overgrew the entire plate. This was avoided by discarding the glass tops and closing the lower half of each Petri dish within two earthenware tops. By sterilizing the lower half within this pair of lids, the plates could be incubated for a number of days in a saturated atmosphere, with satisfactory results.

Examination of Nongravid Uterus.—Three hundred and eighty-nine nongravid uteri were examined microscopically for evidence of infection by *B. abortum*. These uteri fell broadly into two classes. In one group, the uterine walls were more compact and firm to the touch. The outer surface was smooth. In cross section, the outline was more rigidly circular. Both inner and outer surfaces were free from marked congestion. Altogether, 173 uteri of this type were considered normal. In the other group, the uterus was larger, the walls were more flaccid and showed more distinct vascularization. The inner surface usually showed congestion. In cross section, the uterus tended to flatten out. This group, numbering 216, was judged to be from animals that had farrowed more recently than those of the preceding group.

From the normal group, cultures were made of 111 by swabbing the mucosa. In no instance was *B. abortum* found.

In the group of 216 uteri taken from animals more recently parturient, 197 were clean on the inside, free from pus, blood clots and other detritus. *B. abortum* was not found in cultures from the mucosa of 148 of this number. The remaining 19 contained yellowish white lochia. Ten when freshly opened

⁸ Ann. de L'Inst. Pasteur, 1908, 22, p. 541.

gave the odor characteristic of the normal uterus. Microscopically, the lochia showed an abundance of leukocytes, but no bacteria. Cultures proved sterile. This condition probably represented the normal process by which the uterus is cleansed after parturition.

Nine of the nongravid uteri containing lochia were infected with bacteria: Five were filled with a yellowish white lochia of sufficient consistency to round up on the blade of a scalpel. There was no detectable bad odor. Microscopically, this material showed an abundance of polymorphonuclear leukocytes and a number of gram-positive cocci. From 3 were obtained cultures of *Staphylococcus albus*; from 1, a mixture of this organism and a hemolytic streptococcus; and from a fifth a pure culture of a hemolytic streptococcus.

A sixth specimen was somewhat similar in appearance to the preceding, but the lochia had disappeared almost completely and was quite watery. The odor was not bad. The number of leukocytes observed in stained smears was much smaller than in the preceding specimens. A gram-positive coccus was present in the stained smears, and *Staphylococcus albus* was isolated in pure culture.

The results obtained with three other nongravid uteri are interesting because of the isolation of organisms identical in all tests made with *B. abortum* of bovine origin.

Uterus 4 PUR was inflamed and enlarged. The lochia obtained from a sterile incision was darker than that normally found in specimens which were sterile or infected with cocci. The odor was abnormal, but not putrid. Further examination showed the presence of several fetal bones of a size corresponding to that of a fetus of about 70-90 mm. Smears of the uterine pus showed many gram-negative bacilli and a few leukocytes. A bacillus possessing the properties of *B. abortum* isolated from cases of abortion in cattle, was isolated in pure culture.

In uterus 24 PUR, a slight amount of watery lochia was present; the odor was normal; smears of the fluid showed many gram-negative bacilli. *B. abortum* was isolated in pure culture.

In the third nongravid uterus, the lochia were dirty yellow and of a disagreeable odor; smears showed mixed infection; *B. abortum* and a gram-positive diphtheroid were isolated by cultural methods.

Data on the occurrence of *B. abortum* in the uterus of swine are limited. The cumulative results of many investigations with cattle point to the udder and to the associated lymph glands as the seat of infection in the nonpregnant animal. During pregnancy the organisms may, and frequently do, invade the uterus. After abortion or parturition at full term accompanied by uterine invasion, the bacilli disappear from the uterus. Schroeder and Cotton⁹ state that after abortion the bacilli may be found in the discharge for 50 days. Subsequently Cotton¹⁰ reported that the organisms commonly disappear from the uterus after abortion or parturition in 2 to 3 weeks. The longest time he found them to persist was 51 days. From the vaginal discharge following abortion in 2 sows, Hayes¹¹ isolated *B. abortum* at 11 and 24 days, respectively. Connaway, Durant and Neuman¹² slaughtered a sow 58 days after an abnormal farrowing that resulted from ingestion of *B. abortum*. In the uterus was found a fragment of skull bone. No special effort was made to isolate abortion bacilli, which had been previously isolated from the right and left horn, respectively.

⁹ Jour. Agric. Res., 1917, 9, p. 9.

¹⁰ Jour. Amer. Vet. Med. Assn., 1919, 55, p. 504.

¹¹ Ibid., 1922, 60, p. 435.

¹² Mo. Agric. Exper. Station Bull., 1921, 187, p. 3.

In the present survey, the swine were sent to slaughter at varying stages of pregnancy or at different times of the intergestation period. The results, therefore, indicate in a broad way, the prevalence of uterine infection by *B. abortum*: 389 nongravid uteri were examined microscopically, and 259 cultured; 19 of the latter number contained purulent matter and *B. abortum* was encountered 3 times in the purulent exudates. In one of these cases, there was retention of fetal bones.

Of the remaining 16 uteri in which a purulent fluid occurred, 10 were found to be sterile, 1 was infected with *Staphylococcus albus* and a hemolytic streptococcus, 1 with a hemolytic streptococcus alone and 4 with *Staphylococcus albus* alone.

Examination of Gravid Uterus.—In all, 289 gravid uteri were examined. From 181, cultures were made of uterine chorionic scrapings and of stomach contents and organs from macerated embryos when found. *B. abortum* was found once in the membranes and tissues of a macerated embryo.

In this instance, the uterus contained 7 embryos averaging 18 mm. in length. Six were normal in appearance and were suspended in a clear amniotic fluid. The chorionic surface of the seventh sac was pale and covered with a thin white film. The embryo was suspended in a flocculent, turbid amniotic fluid, had lost its normal luster and showed signs of beginning disintegration. A small gram-negative bacillus was readily demonstrated in smears of the fluid and the macerated tissue, from both of which was obtained a pure culture of *B. abortum*.

CHARACTERISTICS OF THE ORGANISMS

Culturally all the strains isolated from swine corresponded to the usual tests for *B. abortum* from bovine sources. Two of the strains that were injected into guinea-pigs produced more lesions of the spleen than were given by many of the bovine strains examined, but these lesions were not more marked than were obtained with a freshly isolated culture from bovine source. Schroeder¹³ and Traum¹⁴ have reported that cultures of *B. abortum* from swine were found more pathogenic for guinea-pigs than cultures from cattle, but were alike in other respects.

To study the serologic relationship of these cultures of porcine origin to cultures from cattle, a series of agglutination and absorption tests were made, using the serum of rabbits, one immunized against a bovine strain of *B. abortum* and one against a porcine strain. No marked difference between the different strains was observed (tables 1 and 2).

¹³ Jour. Amer. Vet. Med. Assn., 1922, 60, p. 542.

¹⁴ Annual Rpt. Univ. of Calif. Div. Vet. Sc., 1920-1921, p. 6.

The sources of the strains were as follows:

Strain DP	Swine	Purdue University
Strain 281	Bovine	Rockefeller Institute
Strain 805	Bovine	Michigan Agricultural College
Strain 58PUP	Swine	Union Stock Yards
Strain 24PUR	Swine	Union Stock Yards
Strain 4PUR	Swine	Union Stock Yards
Strain K	Bovine	University of Kentucky Agricultural College

In table 2 are shown the results obtained in the 1:100 dilution after absorption. No trace of agglutination occurred in any higher dilution.

TABLE 1

AGGLUTINATION

The figures give highest dilution giving complete agglutination

Strains	Swine DP	Bovine K
Bovine 805	800	400
Bovine 281	400	800
Swine DP	800	1600
Swine 58PUP	400	800
Swine 24PUR	800	800
Swine 4PUR	800	1600

TABLE 2

AGGLUTINATION IN 1:100 DILUTION AFTER ABSORPTION

	Serum of Swine Strain DP Absorbed by:		Serum of Bovine Strain K Absorbed by:	
	Swine DP	Bovine K	Swine DP	Bovine 281
Bovine 805	Trace	0	0	Trace
Bovine 281	Trace	0	0	0
Swine DP	Trace	Trace	0	0
Swine 58PUP	Trace	0	0	0
Swine 24PUR	0	0	0	Trace
Swine 4PUR	0	0	0	Trace

EXAMINATION OF MACERATED EMBRYOS

In the gravid uterus, I observed frequently 1 and in one instance 2, partly digested embryos that with their associated membranes were microscopically and culturally sterile. The remaining embryos in such cases were apparently normal. All told, 27 such instances were noted, or 9.4% of the total number of gravid uteri examined. The length of the smallest embryo found undergoing disintegration was 2.5 cm. and of the largest one was 18.2 cm. In one case in which 2 macerated fetuses were found, they were in adjacent positions.

Maceration in all stages was observed. Some embryos were chocolate brown and shriveled, while others showed only slight discoloration. When the flesh was teased with a needle, a difference in consistency was noted. In general, death of one young pig in a uterus with no apparent abnormalities in the others

was found at almost any stage of pregnancy. The odor of this macerated tissue was no different from that of the remaining parts. In smears made from the macerated tissue, organs and fluids, bacteria could not be demonstrated. Although colon bacilli and staphylococci developed in small numbers on some plates, *B. abortum* was never found.

Hayes¹¹ reported a number of cases in which sows giving negative agglutination reactions to *B. abortum* farrowed dead pigs. In a discussion of Hayes' paper, Connaway¹⁵ said: "It is probable that the nonreactors which farrowed good litters were not infected, while the probabilities are that all nonreactors which farrowed some dead and some living pigs were carriers of abortion infection, but that at the time the tests were made, the antigenic action may have been very weak and agglutinins may not have been present in sufficient amounts to show the reaction."

As it was not possible to obtain samples of blood of the sows from which I obtained my specimens, the correlation of the reaction of an animal to the presence of dead pigs in the uterus could not be determined, but the negative cultural findings of the membranes, fluids and organs or tissue of 27 macerated embryos indicate that *B. abortum* was not an active factor in the cases examined.

AGGLUTINATION REACTIONS OF SWINE

A further attempt to determine the extent of the infection of swine by *B. abortum* was made by the use of the agglutination test.

The reaction has been recognized to have distinct value in the diagnosis of infection of cattle. Surface¹⁶ states that agglutination in dilutions of 1:100 and higher may be regarded as practically certain evidence of infection. Agglutination of 1:50 may be obtained with the blood of some noninfected cows. Of the cows whose blood agglutinates *B. abortum* in dilutions between 1:20 and 1:100, some may be infected and some may not. Seddon¹⁷ states that animals which have shown clinical signs of the disease (i. e., have aborted) gave a reaction with 0.01 c.c. of serum in 1 c.c. of suspension. Smillie, Little and Florence¹⁸ concluded that a dilution of 1:40 or lower is negative. Schroeder¹³ states that even a very low agglutination reaction must be looked on with suspicion. The general estimate of the value of the agglutination reaction is that it indicates present or past infection but not necessarily a past history of abortion. According to Fitch,¹⁹ it is a valuable agent in determining herd infection. The data on the reaction of the blood of swine to *B. abortum* relate primarily to infected herds. Connaway, Durant and Neuman⁴ found that 21 of 29 pure bred sows in suspected herds gave positive complement-fixation tests. Doyle and Spray⁶ reported positive agglutination reactions in dilution with blood from 57 of 92 sows in herds in which abortion had occurred.

In this work, the agglutination reaction was applied to the blood of swine killed at local abattoirs. Tests were made on the blood of 625 animals to determine the presence of agglutinins for *B. abortum*. Specimens were collected at the time of bleeding. The serum was agglu-

¹⁵ Jour. Amer. Vet. Med. Assn., 1922, 60, p. 448.

¹⁶ Ky. Agric. Exper. Station Bull., 1912, 166, p. 362.

¹⁷ Jour. Comp. Path. and Therap., 1919, 32, p. 1.

¹⁸ Jour. Exper. Med., 1919, 30, p. 341.

¹⁹ Jour. Amer. Vet. Med. Assn., 1920, 56, p. 459.

tinated against a typical strain of the organism, the first readings being made after 2 hours at 37 C. and the final readings after the suspensions had been held over night in the icebox. Of the total number examined, 435 specimens were from sows and 190 were from barrows. Of 435 samples of sows' blood, 64 or 14.7% gave a positive agglutination in a dilution of 1:50 or higher. Of these 64 findings, 39, or approximately 9%, were in a dilution of 1:100 or higher. The 39 specimens that agglutinated the antigen in a dilution of 1:100 or higher fell into groups as shown in table 3.

TABLE 3
AGGLUTINATION OF B. ABORTUM BY SWINE SERUM

No. of Specimens	Highest Dilution at which Serum Caused Complete Agglutination
8.....	1: 100*
11.....	1: 100
15.....	1: 200
3.....	1: 400
1.....	1: 600
1.....	1: 800

* Partial agglutination only.

Of 190 specimens of barrows blood tested, 10 or 5.3% agglutinated the antigen in a dilution of 1:50 or higher and 5 or 2.6% in a dilution of 1:100 or higher. One of the 5 gave a reaction in 1:200 and 1 in 1:400 dilution.

INFECTION OF SWINE BY FEEDING B. ABORTUM

In previous work it was found that while approximately 9% of samples of sow's blood agglutinated B. abortum in a dilution of 1:100 or higher, the organism could rarely be found in the uterus. The object of the present work was to study on a limited scale the susceptibility of swine to bovine strains of B. abortum and to determine, if possible, the localization of the organism in the animal body.

Good and Smith³ produced abortion in a pregnant sow in 17 days by intravenous injection of a porcine strain. A second sow aborted 19 days after an initial feeding of the washings of 2 slants, followed by 5 slants 7 days later. B. abortum was isolated from the heart, liver and stomach contents of the embryo in the first instance, and from the stomach contents in the second. The blood of the adult pigs agglutinated a known culture of B. abortum completely in a 1:100 dilution and partially in a 1:150 dilution.

Connaway, Durant and Neuman^{12,20} tried intramuscular, intravenous and intra-axillary injections, intravaginal douchings and feeding cultures of B.

²⁰ Mo. Agric. Exper. Station Bull., 1921, 172, p. 45.

abortum. Two of the injected pigs aborted; one, which became a reactor, farrowed 1 dead and 6 healthy pigs, and 1 farrowed 4 dead pigs. The 2 sows inoculated intravaginally developed no reaction and did not abort. Feeding of aborted swine fetuses and of cultures from aborted swine fetuses produced positive results.

Hayes¹¹ fed cultures from swine to young sows. The animals became positive reactors but did not abort. *B. abortum* was isolated at farrowing in some instances, but not in all.

In a general discussion of the work being done by the U. S. Bureau of Animal Industry on abortion, Schroeder¹³ states that their results indicate that swine are strongly resistant to the ordinary porcine strains.

FEEDING EXPERIMENTS

In this work, a pregnant sow, 16 months old, with a history of one previous normal parturition, was obtained. Agglutination tests of the blood serum were negative with a suspension of *B. abortum* in dilutions of from 1:10 to 1:80. Both cattle and swine at the farm from which the pig was purchased were said to have been free from abortion. The date of the breeding was May 24, 1921.

On July 11, the washings of 10 agar slants of a culture of *B. abortum* of bovine origin were fed. A bovine strain was selected because of the frequent occurrence of this organism in raw milk from infected cows. Injected into a pregnant guinea-pig, the culture had produced abortion in 10 days. August 1, the blood serum of the sow agglutinated the same strain of *B. abortum* completely in a dilution of 1:40 and partially in a dilution of 1:160. Serum obtained on Aug. 16 and on Aug. 25 agglutinated this strain completely at 1:320.

On Sept. 16, 1921, the day for the termination of gestation, the blood serum agglutinated *B. abortum* completely in a dilution of 1:80 and partially in a dilution of 1:160. No trace of agglutination occurred at 1:320.

On the evening of Sept. 16, the sow farrowed 5 live, and seemingly healthy pigs and 1 macerated fetus. Four were suckling when found, and the fifth was removed before it had a chance to suckle.

A bacteriologic examination of material obtained at farrowing was made by cultural tests and guinea-pig inoculations to determine whether infection by *B. abortum* existed. Cultures were made on sheep serum agar plates. Guinea-pigs used for inoculation were bled from the heart and the serum tested for its agglutination reaction against *B. abortum*. The animals were tested also cutaneously by injecting intradermally 0.1 c.c. of a killed suspension of this organism as used by Reichel and Harkins²¹ and Stafseth.²² As a control injection, a killed suspension of typhoid bacilli was used. The reactions were compared with those given by guinea-pigs known to be infected with *B. abortum*. Observation was continued 3 days. Animals giving no trace of agglutination in dilutions of 1:10 or over and with proved negative skin reactions were used. Stock was prepared a couple of weeks in advance.

PA-P1: A female pig which was suckling when found was removed and killed at once. The blood serum failed to agglutinate *B. abortum* in dilutions

²¹ Jour. Amer. Vet. Med. Assn., 1917, 50, p. 847.

²² Mich. Agric. Coll. Exper. Station Tech. Bull., 1920, 49, p. 14.

ranging from 1:10 to 1:80. A retest gave the same result. The blood serum of the sow on the day of farrowing had agglutinated this organism in dilutions up to 1:160.

Stomach contents, macerated spleen, kidney and liver tissue of this pig were seeded on serum agar plates in duplicate sets. One set was incubated in ordinary atmosphere and one set in closed jars in a modified atmosphere, at 37 C. for 10 days. Examination was made on the fifth and the tenth days, respectively. The plates of liver, spleen and kidney tissue gave no growth with the exception of a few contaminating organisms; plates of the stomach contents were thickly overgrown with organisms evidently ingested. *B. abortus* was not found. Smears of stomach contents stained with carbol fuchsin and Gram's stain were negative.

Two guinea-pigs were injected intraperitoneally with the stomach contents of the young pig, the one receiving 1.5 c.c. and the second, 0.75 c.c. The first guinea-pig died in 7 days of a peritonitis. The second one gave a doubtful cutaneous test at 8 weeks and a negative one at 12 weeks. At necropsy, the organs of this guinea-pig appeared negative. Culture of spleen, testes and kidney, and composite suspension of liver, spleen and kidney injected into a second guinea-pig gave negative results.

PA-P5: This pig was killed before it had suckled. The blood serum gave no agglutination in dilutions of 1:10 to 160. Macerated portions of liver, spleen and kidneys, stomach and intestinal contents were plated on serum agar plates which were incubated 6 days in modified atmosphere. *B. abortus* was not found. Smears of the stomach contents stained with carbol fuchsin and with Gram stain were negative.

Further tests for the presence of *B. abortus*, made by injecting stomach contents of pig PA-P5 into 3 guinea-pigs and saline emulsion of liver into a fourth guinea-pig were negative.

PA-P6: Macerated embryo showed marked signs of intra-uterine digestion. A microscopic examination of the body tissue showed no organisms. Cultures from the stomach, liver and spleen were negative. A guinea-pig was injected intraperitoneally with 4 c.c. of a saline emulsion obtained by mixing stomach, liver and spleen tissue. The cutaneous test as well as cultures of the spleen, liver, kidney and testes made at necropsy were negative.

Examination of Placenta.—The placenta at the time of expulsion was caught in sterile cans. Appearance, normal; scrapings from the surface of each placenta were inoculated on serum agar plates with negative results. Scrapings of the placentae were injected into the peritoneal cavity of a guinea-pig, but the animal died in 4 days of peritonitis.

Although infection of the dam was shown by the positive agglutination titer in fairly high dilution, the results of cultures and of guinea-pig inoculation indicate that there was no uterine invasion by the abortion bacillus during pregnancy. A similar phenomenon has been known to occur in cattle and has been demonstrated by Hayes¹¹ to occur in swine. Connaway and his associates¹² have found that when uterine invasion occurs, the colostrum has been infected also. The only test made on the colostrum in this work was on that forming part of the stomach contents of the young pig found sucking just after birth. The results were negative.

A further attempt was made to determine whether the milk serum after farrowing contained agglutinins for *B. abortum* and whether the organism could be recovered from the mammary gland. Owing to the unusual sensitiveness of the dam, only 2 to 5 c.c. of milk could be obtained at a time. The agglutination tests with the milk serum were made according to the method described by Cooledge.²³

Examination of Milk.—On Sept. 20, 1921, four days after farrowing, 2 c.c. of milk were obtained; this was diluted with 2 c.c. of salt solution and centrifuged for 30 minutes at high speed. The upper 2 c.c. were used for agglutination and the lower half injected into a guinea-pig. Agglutination tests on the milk serum and guinea-pig inoculation were negative. The blood serum of the sow, 4 days before the specimen was obtained, gave a reaction complete in 1:80 dilution and partial in 1:160. On Sept. 27, similar tests with 5 c.c. of milk were negative, the reaction of the blood serum remaining the same. On Nov. 11, the milk serum was negative, but the reaction of the blood serum had dropped to 1:40.

These results, being negative, are open to the criticism that small amounts of milk were used for injection into guinea-pigs. However, combined with the drop in titer of the blood serum at farrowing followed by a persistent low agglutination reaction of the blood, and the absence of demonstrable uterine invasion, they point to a mild, and in all probability a declining, infection.

Results After Parturition.—As it was noted that the agglutinating power of the blood serum for *B. abortum* had decreased from 1:320 before farrowing to 1:40 after farrowing, further feeding of suspensions of the culture previously used was begun to determine what change would be produced.

Fifteen slants of *B. abortum* were fed 77 days after farrowing, 24 slants 99 days after farrowing and 24 slants 105 days after farrowing. Blood serum obtained the day before the last feeding of *B. abortum* and 11 days after this feeding gave complete agglutination only in a 1:20 dilution.

On Jan. 13, 1922, approximately 6 months after the feeding of the first cultures and 4 weeks after the feeding of the last cultures, the animal was slaughtered. The blood serum at this time gave complete agglutination in a 1:20 dilution and partial in a 1:40 dilution.

Salt solution emulsions of liver, ovaries, spleen, mesenteric lymph glands, kidney, uterine washings, nonlactating portion of the mammary gland and lactating portion of the mammary gland, kept functioning by allowing 2 of the young pigs to suckle, were injected into previously tested guinea-pigs. The cutaneous reactions at 8 weeks were negative for *B. abortum*. No lesions were found at necropsy. Cultures of the spleens of the guinea-pigs were negative.

These negative findings and the constantly low agglutination reaction indicate not only the elimination of the original infection, but also elimination of the organisms later ingested. Sexually mature sows, as

²³ Jour. Agric. Res., 1916, 5, p. 871.

a rule, according to Connaway and his coworkers,¹² retain the abortion infection indefinitely. Some, however, lose the reaction and presumably the infection. Pregnancy also was not found a necessary condition to infection. Many of their results were based on naturally infected animals. With the single experimental animal used in the foregoing, the first feedings were given during pregnancy and the later ones after farrowing at a time when the serum titer had dropped to a stationary level. Whether the failure to obtain an increased agglutination reaction indicates a loss of invading power of the culture, an individual resistance, or an immunity to second infection after the elimination of a first, can be determined only by work with a much larger number of animals.

FEEDING EXPERIMENTS WITH YOUNG PIGS

Additional work on the susceptibility of pigs sexually immature was undertaken. The 3 young pigs used were the offspring of sow PA that had developed agglutinins for *B. abortum* in the blood following ingestion of a culture of this organism of bovine origin but that had subsequently carried her young to full term without invasion of the uterus. Following parturition, the titer of her serum fell from 1:320 to 1:20 and was not increased by subsequent feedings. There was evidence that at the time of farrowing the infection was declining.

In the following experiments, one gilt and one young boar were fed culture P. 35 isolated several months previously from the infected uterus of a cow. A second gilt was fed a culture of the same strain fed the dam.

Pig PA-P3: Young female. Date of birth Sept. 16. Agglutination reactions of blood serum obtained Nov. 2 and Nov. 28 were negative in dilutions from 1:20 to 1:80. Beginning Dec. 1, at 2½ months of age and extending over a period of 4 months, the animal was fed, at intervals of 4 to 7 days, a total of 159 slants of *B. abortum*. The culture, P35, had previously been isolated from the infected uterus of a slaughtered cow.

The blood serum of the pig at times gave a partial agglutination reaction in a 1:20 dilution but never a complete reaction until the 4th month after the first feeding, when complete agglutination occurred in a 1:20 dilution and partial in a 1:40. Feeding of organisms was discontinued. Six weeks later the reaction was negative in 1:20 and 1:40 dilutions.

At the time of the last feeding, the animal was approximately 6½ months old. The amount of culture fed represents 159 slants given over a period of 4 months, beginning at 2½ months of age.

Two other pigs raised from the same dam, 1 a boar and 1 a gilt, were fed a total of 40 slants each, 2 slants at a time over a period of 32 days beginning at 4½ months of age. The boar received culture P35 as did the first gilt; the second gilt was fed the same strain given the dam. Before feeding, the agglutination reaction of the boar, at times, was positive in a dilution of 1:10

and of the gilt in a 1:20 dilution but in none higher. Fifty-five days after the first feeding, the reaction of the boar was slightly positive in 1:10 and of the gilt complete in a 1:20 and partial in a 1:40 dilution. One hundred and thirty-one days after the first feeding, the reaction of the boar was negative in dilutions of 1:10 to 1:80, and of the gilt, positive in 1:20 and negative in 1:40 and 1:80.

Connaway and his associates¹² report that young pigs born of infected dams and giving positive reactions in most instances, lost the reaction, about weaning time and did not again become reactors, even though they had suckled milk infected with the bacilli. They state further that no reinfection of pigs that have become negative to the abortion test has occurred. The idea is implied that subsequent exposure did not occur. One instance is cited of a gilt which had become negative to the complement fixation test and remained so at the time of first breeding. Hayes¹¹ has observed that 50% of a lot of pigs placed at weaning time in an infected environment presumably among other infected pigs, gave agglutination reactions to *B. abortum* at 6 months.

In the work reported here, one gilt developed a slight increase in the agglutination reaction above that obtained in the control tests, as a result of being fed 159 slants of a bovine culture over a period extending from 2½ to 6½ months of age. This was considered as a possible sign of beginning infection, but at 9 months of age there was no evidence of infection as determined by the agglutination test. A boar fed 40 slants of the same culture over a period extending from 4½ to 5½ months of age, gave no change in reaction up to 9 months of age. The second gilt fed 40 slants of the same bovine strain used previously with the dam, over a period extending from 4½ and to 5½ months of age, gave a slight increase in the agglutination reaction at 6 months of age, but a normal reaction at 9 months.

These results indicate a high resistance of the young animals to oral infection by the cultures used.

INFECTION OF RABBITS WITH *B. ABORTUM*

The question whether infection by *B. abortum* occurs more readily through the alimentary tract or through the genital tract is important in the cattle and swine industry in connection with herd sanitation. In contradiction to the opinion that the genital tract is a common avenue of infection of cattle is the statement of Schroeder¹³ that the investigations of the Bureau of Animal Industry do not warrant the assumption that cows are infected by way of the vagina and uterus or that the bull spreads the infection.

The incrimination of the bull as a systemic or mechanical carrier is based on the fact that some animals have been discovered with infected

genitalia, that intravaginal inoculation of pregnant cows has produced abortion and that herd infection has resulted from the introduction of infected bulls into abortion free herds.

The data on the possibility of infection in swine by the male, either as a mechanical or systemic carrier, are not so extensive as those on cattle. Doyle and Spray⁶ reported a positive agglutination reaction with the blood of a boar in a dilution of 1:500. Connaway¹² and his associates have found 4 positive reacting boars in infected herds, 2 of which had diseased testicles. Two other nonreacting boars were bred to reacting sows and both became permanent reactors, whether by copulation or ingestion of infected material was not known. Hayes¹¹ bred 2 gilts to a naturally infected boar. Although both farrowed some dead pigs with the living ones, *B. abortum* was not isolated at farrowing time in one case and cultures obtained from the second were not identified. The reaction of the sows remained negative. An emulsion of the testicles of the boar to which the sows were bred gave negative results by guinea-pig inoculation.

The experimental work with rabbits was performed to determine whether these animals were more susceptible to invasion by *B. abortum* through the genital tract or through the alimentary tract. In selecting these animals, it was realized, of course, that what might happen in one species of animal might not happen in another. The method used was that of continued feeding and implantation of suspensions of *B. abortum* on the genitalia of different lots of rabbits.

Experiments.—All rabbits used were tested twice for the agglutinating power of their blood serum with a suspension of *B. abortum*. An interval of 10 days was allowed to lapse between the last bleeding and the first exposure to cultures. Those that were fed received 0.1 of a slant on two successive days. After a 6-day interval, feeding of 0.1 of a slant daily was resumed and continued until the 21st day, with exceptions noted. The animals were tested 21 and 27 days after the first feeding, for the presence of agglutinins for *B. abortum*. Exposure to infection through the genital tract was obtained by irrigation with a saline suspension of the organism every other day for periods up to 28 days. The animals were tested on the 21st day, except where noted, and tested again 35 to 50 days after the first exposure.

Feeding Adult Rabbits.—Eight male rabbits were fed in the manner stated, 4 with the strain from cattle that had been used with 2 of the gilts previously noted, and 4 with a strain from swine. One fed a bovine strain died; 6 showed agglutinins in a dilution of 1:320 and 1 fed a porcine strain, gave a reaction in 1:80.

Eight female rabbits were divided into similar lots and fed a culture of the same organism.

One, fed a bovine strain, gave birth to full term young on the 26th day. Agglutination test on the 27th day was negative. Subsequent feeding for 11 days caused the production of a positive reaction in a dilution of 1:40, 19 days after the beginning of the second feeding.

A second rabbit, fed a bovine culture, 11 days after the first feeding, gave birth to 4 apparently full term young, which were found partly eaten. Guinea-

pigs inoculated with material from the stomach and organs of 2 of the young rabbits were negative for *B. abortum*. An agglutination reaction at 21 days was positive in dilutions of 1:30.

Three of the rabbits, 2 fed a bovine and 1 a porcine culture, developed agglutination reactions ranging from 1:80 to 1:640. None of this group, as far as is known, was pregnant.

One rabbit, fed a porcine strain, aborted on the 15th day. *B. abortum* was obtained from the aborted material by culturing. The serum of the rabbit agglutinated *B. abortum* in a dilution of 1:320. The remaining 2 animals died of snuffles.

Implantation of Organisms in the Genital Tract.—Five female adult rabbits were given intravaginal douchings of *B. abortum* every second day for 28 days. These were selected as nonpregnant females, and, as far as is known, they neither gave birth to young nor aborted at any time while under observation. The material was injected with a medicine dropper, the point of which was dipped into petrolatum before implantation. Surplus fluid was removed by cotton. The animals were watched for the first few minutes to prevent licking of the genitals. They were kept without food for 24 hours before each treatment, but were fed immediately on being returned to the cages. Invariably they began eating vigorously, making no attempt to lick themselves.

Four of the 5 animals remained negative through the experiment and were negative at the 40th day. The 5th rabbit developed such a bad case of snuffles and diarrhea simultaneously that the intravaginal douchings were discontinued. When recovery seemed doubtful, the animal was bled on the 16th day. It died from a hemorrhage into the pericardium. The agglutination reaction was positive to *B. abortum*, complete in a 1:40 dilution and partial in a 1:80 dilution. As the rabbit became badly soiled during the experiment, particularly about the face, there is a possibility that material containing the organisms may have been smeared on the face and subsequently ingested.

Another lot of 5 female rabbits was treated in a similar manner by intravaginal injections. During the intervals between treatments, they were placed with males and were allowed to remain for 24 hours. Whenever the female repelled the male, she was removed. Two of the rabbits became pregnant and gave birth to fully developed young. None of the animals became reactors to *B. abortum* during a period of observation of 50 days.

A lot of 4 males was treated every other day by having the sheath pulled forward over the penis and filled with a suspension of *B. abortum*. Surplus suspension was taken up with cotton. This was continued for 24 days, except in the case of 1 rabbit, which died on the 15th day. The heart blood of this rabbit was taken up in saline and tested. No agglutination reactions were obtained either with the blood of this rabbit, or of the others held for 35 days.

Feeding of Immature Rabbits.—A lot of 5 young female and 5 young male rabbits, ranging in weight from 497 gm. to 690 gm., was fed according to the same schedule as the foregoing rabbits. An epidemic of snuffles began among the animals and wiped out the entire lot in 24 days. Of those living at least 16 days, 4 females and 2 males developed positive reactions. The dilutions ranged from 1:160 to 1:320. The incidence of infection among the surviving animals, therefore, was 100%.

Control Feedings.—The experiments described above were continued, with the interruption noted, over a period of 21 days, making a total of 15 feedings of 0.1 slant each. To determine the effect of single feedings, 2 adult rabbits,

1 male and 1 female received the growth of 1 slant of culture 35 at 1 feeding. One gave a reaction in a dilution of 1:160 when tested at 33 days and the other one a reaction in a dilution of 1:80 at 37 days.

To determine the effect of smaller amounts of organisms, 3 adult rabbits, 2 males and 1 female, each received 0.1 of a slant of culture 35 on 3 successive days. At 18 days after the first feeding, all were bled. One gave a positive reaction in a dilution of 1:320, one a weak reaction in 1:80; and one a strong reaction in 1:80 dilution. An additional lot of 1 male and 2 female adult rabbits was fed suspensions of culture 35 killed at 60 C. for 1 hour. Fifteen feedings of 0.1 slant each over a period of 30 days did not cause the production of agglutinins in dilutions ranging from 1:40 to 1:320. The tests were made at 21 and 35 days.

A comparison of results shows that the rabbits fed live cultures of *B. abortum* were readily infected in practically all cases. A frequent implantation of organisms on the genital tract failed to cause the development of agglutinins except in one case in which the animal became ill from both snuffles and diarrhea. Young rabbits were infected by feeding with about the same ease as the older ones, and in this respect differed from the young pigs in susceptibility.

These results indicate a much higher resistance of rabbits to infection through the genital tract than was observed by Sanderson and Rettger ²⁴ in a recent paper. A special effort to exclude oral infection by licking the genitals was made by withholding food for 24 hours before exposure and then feeding the animals immediately afterward, in order to divert their attention away from the treatment. As all my strains were at least several months old, a difference in the quality of the organisms must be considered.

SUMMARY

Organisms culturally and serologically identical with *B. abortum* (Bang) were isolated 3 times in 389 nongravid swine uteri examined microscopically, of which number cultures were made in 259.

This organism was isolated once from a macerated fetus and associated membranes in an examination of 289 gravid swine uteri, of which number cultures were made in 181.

The agglutination reactions of the blood serum of 435 sows slaughtered at abattoirs were positive in a dilution of 1:50 in 14.7% of the specimens and in a 1:100 dilution in 9% of the specimens. Of 190 specimens of barrow's blood, 10 or 5.3% were positive in a 1:50 dilution and 5 or 2.6% in a 1:100 dilution.

Feeding of suspensions of *B. abortum* to a pregnant sow on the 48th day of gestation resulted in the production of agglutinins but not in

²⁴ Jour. Infect. Dis., 1923, 32, p. 181.

abortion. Five healthy pigs and one macerated fetus were farrowed at the termination of the normal gestation period. *B. abortum* was not isolated from the fetal membranes, macerated fetus or from the stomach contents and organs of the healthy pigs. The agglutination reaction of the dam declined at or shortly before farrowing and was not increased by further feeding of the organism to the nonpregnant animal. Elimination of the initial infection and of subsequently ingested organisms was indicated by failure to recover *B. abortum* from the liver, ovaries, spleen, mesenteric lymph glands, kidney, intra-uterine surface, lactating and nonlactating mammary glands, 6 months after the feeding of the last culture.

Two gilts, 1 fed cultures of *B. abortum* over a period of four months, beginning at 2½ months of age, and 1 fed cultures for 32 days, beginning at 4½ months of age, gave slight and inconclusive increases in their agglutination reactions at 6½ months. At 9 months, the reactions were the same as those given by the animals before they were fed the organisms. A boar fed cultures of the organism for 32 days beginning at 4½ months, showed no change in the agglutinating power of its blood serum up to 9 months of age.

In experiments on the avenue of infection of rabbits by *B. abortum* as determined by the agglutination test, it was found that agglutinins developed readily in male and female rabbits as a result of ingestion of *B. abortum*. With one exception, agglutinins did not develop as a result of frequent implantation of the organism in the genital tract.

STUDIES ON SEROLOGIC CLASSIFICATION OF B. BOTULINUS

1. PREPARATION OF ANTISERUM

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During the past few years a study of the serologic classification of over 100 strains of *B. botulinus* has been in progress in this laboratory. On account of the extreme toxicity of the organism, the problem of immunizing a large series of animals promised to be a difficult one. We are glad to report, therefore, that with a few exceptions, antiserum of high titer has been produced by the intravenous inoculation of washed and heated cultures.

An 18-24 hour growth in double strength veal infusion broth containing 0.5% NaCl, 0.5% dibasic potassium phosphate, 1.0% Witte's peptone, and 2.0% glucose, was centrifugalized at high speed and washed two times in from 50 to 75 c.c. of sterile salt solution. The sediment was then resuspended in fresh salt solution and heated for one hour in a water bath at 60 C. Tricresol in the proportion of 0.2% was occasionally added as a preservative, that is, when the preparation had to be kept for a few days. Ordinarily, fresh suspensions were used for each inoculation. The number of organisms was estimated either by a direct bacterial count in the Halber chamber or by diluting a measured volume to the same opacity as a standardized barium sulphate suspension.

To produce antiserum, young rabbits, weighing preferably from 1,500-2,500 gm., were inoculated with definite amounts of the suspension just described. In 1920, when the problem was first attacked in this laboratory by Nelsine Marion Neilson, 4 different methods of immunization were used. Later the work was continued with the following 2: The first one, which will be referred to as method 1, is similar to the one recommended by Hine¹ for the rapid production of meningococcus antiserum. It consists in giving 3 intravenous inoculations of antigen at hourly intervals on the 1st day, followed by a second larger dose on either the 5th or 6th day. The second method is the one used by Tulloch² for the production of tetanus antiserum. He starts with one dose and follows it with a second larger one on the 5th

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¹ Jour. Roy. Army Med. Corps, 1915, 25, pp. 424 and 693.

² Proc. Roy. Soc. Lond., B, 1917-1918, 90, p. 145.

day. Our animals were bled every few days from the ear vein so that the agglutinin content of the serum could be determined. When such determinations had to be made on inoculation days, the blood was drawn before the injection was made.

The antigens for carrying out the agglutination tests were made from bacilli grown on the medium previously described. An 18-24 hour culture was washed twice in salt solution and then resuspended

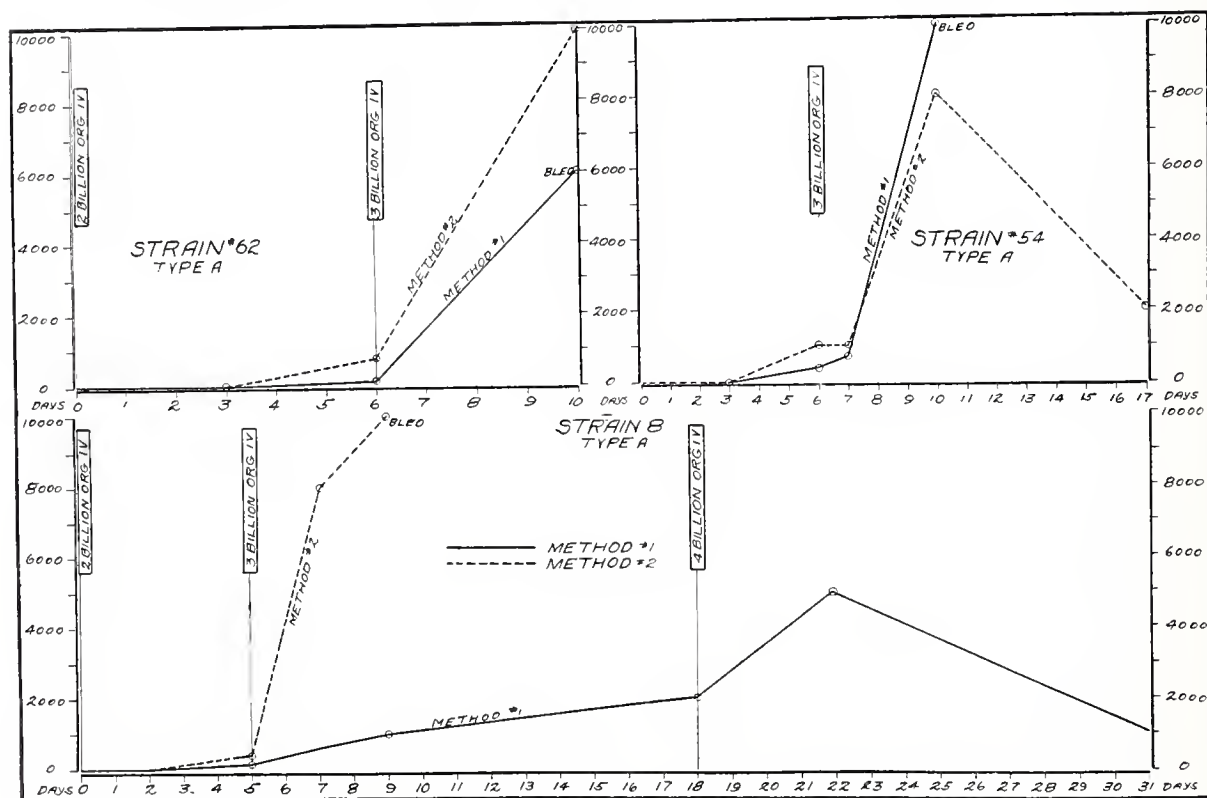


Chart 1.—A comparison of two methods used to produce *B. botulinus* antiserum.

in sterile salt solution to which 0.1% formalin had been added. The preparation was diluted so as to contain 250 million bacilli per c.c. Antigens prepared in this way were stored in the ice chest, and it was found that they would maintain their properties over long periods of time.

All of the tests were carried out in small tubes measuring $\frac{3}{8}$ by 3 inches. The serum was diluted in proportions of 1:10, 1:100, and 1:1,000, and 0.25, 0.16, 0.12, 0.10, and 0.05 c.c. of each dilution was measured into a series of tubes. One c.c. of antigen was added; the tubes were placed in an incubator at 35 C. and allowed to remain at that

temperature for 2½ hours. The results were always read immediately on removal from the incubator, but a check reading was made after the tubes had stood at room temperature over night. Little variation, however, could ever be found between the two readings. The highest dilution causing a clumping of the organism was taken as the index of the titer of the antiserum.

The graphs shown in chart 1, based on the results obtained in a few typical experiments, show how the titer of the antiserum varies

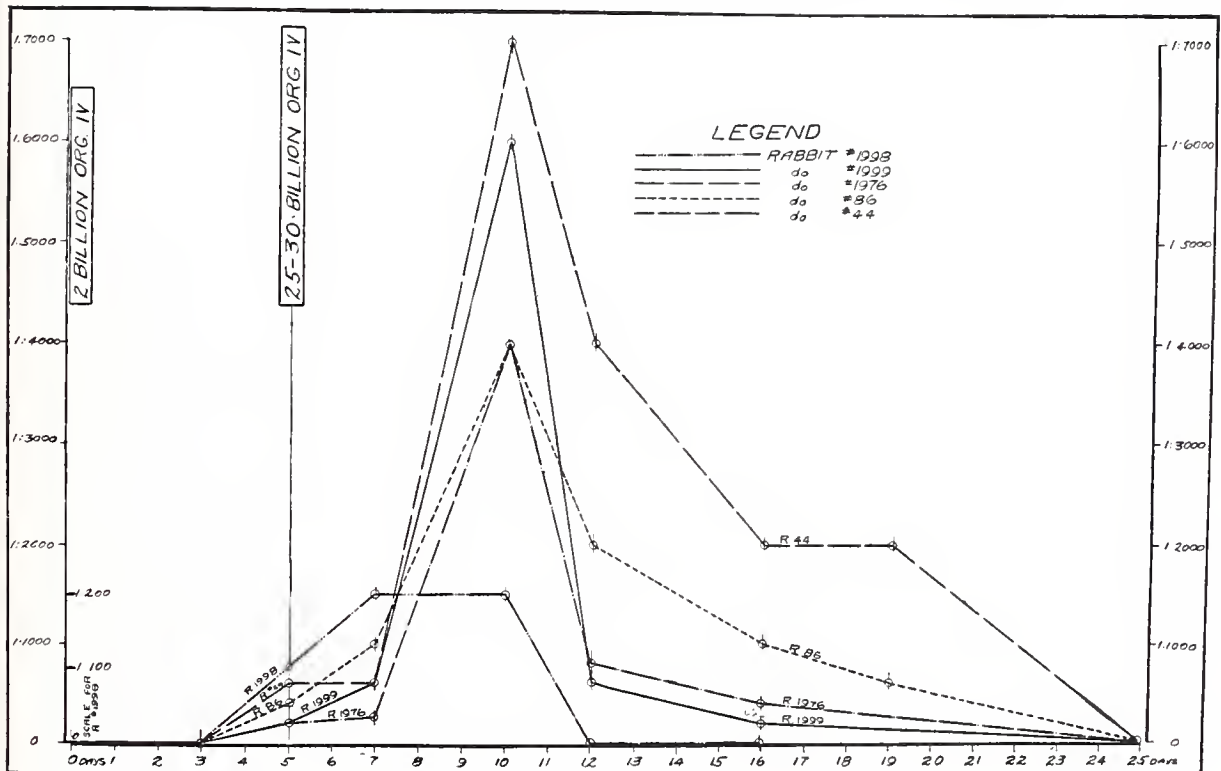


Chart 2.—The production of agglutinins in the serum of rabbits inoculated with *Bacillus botulinus*. Strain 38 Type A.

from day to day. Some agglutinins are practically always present on the 5th day, and may even be found on the fourth. One of the curves, R 1998, in chart 2 shows that the first injection (with method 2) does not lead to the production of much agglutinin, and what is produced disappears rapidly if the second inoculation is not made. Following the second inoculation, the titer of the antiserum increases steadily until the 10th day, when the maximum amount of agglutinin is generally found. The curve then falls off steadily, and after from 24 to 28 days, no more of the agglutinin can be detected. In some cases when, either because of individual peculiarities of the rabbits or because of peculiarities in

the strain, the titer remained low after 2 inoculations, from 1 to 3 more were made at intervals of approximately 1 week. Although occasionally a 3rd injection brought about the formation of a good antiserum, as shown in chart 1, R 66, we feel that ordinarily such injections are hardly worth while. As will be noted in chart 1, method 2 gave generally the best results. Given a good rabbit, one can usually get a serum with a titer between 1:4,000 and 1:10,000. As it is also simpler and takes less time than does method 1, we early adopted it to the exclusion of the other.

A total of 69 rabbits were immunized with various strains of *B. botulinus* according to the several different methods, but as just stated most of the work was done with method 2. Forty-six of the animals responded to immunization satisfactorily; 8 responded poorly, and 15 did not respond at all, or so feebly that the results were considered negative. Eight of these, however, died so early that it is impossible to say whether or not they would eventually have responded to immunization. The other 7 were observed for longer periods of time.

During the course of immunization, or some time after the process had been stopped, 26 of the 69 animals died. In 9 cases, death was attributed to the high toxicity of the strain used. In 2 of these, the trouble may have arisen from giving an excessive number of spores. It might have been avoided had we adhered to our rule to discard any suspension showing more than from 2-3 spores to one microscopic field.

In 12 cases, death was attributed to coccidiosis, and in 5 it seemed to be due to intercurrent infections such as pneumonia, empyema or peritonitis. It may easily be, however, that the immunizing process in some of the latter cases was a contributory cause of death. Serum from 10 of the 12 rabbits that died of coccidiosis had already shown a high agglutinin content, while the other 2 had given no response.

In 1921, Bronfenbrenner, Schlesinger and Calazans³ reported that *B. botulinus*, type A, can be differentiated from type B by means of serologic tests. We have been able to confirm these observations, although occasional high cross-agglutinations have been encountered; and we have, furthermore, shown by means of agglutinin and agglutinin absorption tests that the A types can be subdivided into at least 3 and possibly 4 groups, while the B types can be divided into at least 2 groups. The detailed description of these groups and their characteristics will be left for a later paper.

³ Proc. Soc. Exper. Biol. & Med., 1921, 19, p. 21.

EFFECT OF TEMPERATURE ON VELOCITY OF REACTION IN HEMOLYSIS

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The velocities of most biochemical reactions are not easily measured. Unlike many reactions between "pure" inorganic chemicals, the products formed in most biochemical reactions are as difficult to determine quantitatively as are the rates of disappearance of the reacting substances. This is not true, however, when one of the products of a reaction is a colored substance, for then the velocity of the reaction can within certain limits be determined by the rate of increase in the intensity of color of the liquid in which the reaction is proceeding. Hemolysis, whether caused by physical, chemical or biologic means, is a reaction involving the liberation of hemoglobin from the stroma of the cells, and is accompanied by a change in the physical state of the liquid—a change from a turbid, fairly opaque, pinkish liquid to one that is water clear and of various shades of color, from a faint yellow to an intense red, depending on the concentration of the dissolved hemoglobin. It is, therefore, a reaction which can be measured with a fair degree of accuracy.

REVIEW OF THE LITERATURE

In this paper "hemolysis" means only that liberation of hemoglobin from the cell stroma which is caused by the action of amboceptor and complement, and not that brought about by so-called "chemical" and "physical" means. This review therefore covers only articles that bear directly on this problem.

The controversy between Bordet¹ and Ehrlich,² in which the former maintained that an irreversible chemical change takes place in complement fixation, while the latter held that only a simple physical fixation occurs, has not been settled.³ Although the exact nature of the physicochemical reaction in hemolysis is not known, certain of its underlying principles have been studied.

Investigating the effect of temperature on the reaction, Ehrlich and his collaborators⁴ showed that the affinity of amboceptor for red blood cells is much stronger than for complement, so that at 0 C. amboceptor will attach itself to red blood cells but not to complement, although both are present in the same liquid. Neufeld and Haendel,⁵ Sachs and Bolkowska⁶ and Müller⁷

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¹ Ann. de l'Inst. Pasteur, 1898, 12, p. 688.

² Berl. klin. Wchnschr., 1901, 37, p. 453.

³ Bechold: Colloids in Biochemistry and Medicine, 1919, p. 207.

⁴ Ehrlich: Collected Studies in Immunity, 1906.

⁵ Arb. a. d. Kais. Gsndhtsamte., 1908, 28, p. 198.

⁶ Ztschr. f. Immunitätsf., 1910, 7, p. 778.

⁷ Ibid., 1914, 23, p. 306.

found that complement was fixed by a mixture of red blood cells and homologous amboceptor at temperatures ranging from 8 C. to 3 C. Müller also found that it took less antigen to bind a constant amount of complement at 3-7 C. than at 37. This is in harmony with the findings of Dean,⁸ who showed also that at the latter temperature the maximum fixation was obtained in 30 minutes, while at 0 considerable progress was made after the expiration of the second hour. Bronfenbrenner and Schlesinger⁹ came to the same conclusions, and added that if several units of antigen and amboceptor were used, the fixation of 2 units of complement was complete in the first 5 minutes.

The time which elapses before the maximum fixation of complement occurs in any mixture of antigen and antibody depends on the relative amounts of antigen and antibody present. An excess of either retards the reaction.¹⁰ Cummer¹¹ and Morgenroth and Sachs¹² have shown that when 2 units of amboceptor are employed instead of 1, either the amount of complement, or, the time necessary for completion of the reaction may be greatly reduced. However, Noguchi,¹³ Kolmer,¹⁴ and Zinsser¹⁵ have proved that exact quantitative relations between amboceptor and complement do not exist, and that these relations are not mathematically proportional. Ehrlich brought out the fact that red blood cells are capable of absorbing many times the amount of amboceptor necessary for lysis, and that the presence of free amboceptor can be demonstrated in the solution after the reaction is complete. There is no demonstrable dissociation when less than 4 units of amboceptor are used (Kolmer¹⁴).

Although the temperature of the reacting mixture influences its extent and velocity, little work was done on the problem of complement destruction at various temperatures before that of Noguchi and Bronefenbrenner,¹³ who found a decrease in hemolytic power equal to 33 to 50% in 30 minutes at 45 C., and 50% in 5 minutes at 50. Kolmer showed that inactivation of guinea-pig serum commences at 40 C., but does not become marked below 50. In a thorough study of the problem Bigger¹⁵ concluded that between 9 and 50 C. the rate of loss is regular, and is in accordance with a definite mathematical expression, and that the higher the temperature the more rapid is the loss of complementing power. Gramenitski¹⁶ showed that if complement is completely inactivated by heating at 56 C., some restoration of its activity occurs on standing at low temperatures (about 7 C). No restoration takes place in 2½ hours at 37 C., hence hemolytic ordinary tests are not affected by the change.

It is apparent that the action of complement is akin to that of enzymes. Complement is therinolabile, and a small amount suffices for the destruction of a relatively large amount of antigen. That it is used up during the process of lysis is not in keeping with its other enzymelike properties. Muir¹⁷ believes that complement is used up in the reaction, while Liefmann and Cohn¹⁸ maintain that its appearance is due to fixation by the end products of hemolysis, destruction by dilution, and prolonged preservation at relatively high

⁸ J. Path. & Bacteriol., 1917, 21, p. 193.

⁹ Proc. Soc. Exper. Biol. & Med., 1917, 14, p. 139.

¹⁰ Zinsser: Infection and Immunity, 1919, p. 162.

¹¹ Manual of Clin. Lab. Methods, 1922, p. 140.

¹² Berl. klin. Wchnschr., 1902, 39, p. 817.

¹³ J. Exper. Med., 1911, 13, p. 229.

¹⁴ Am. J. Syph., 1921, 4, p. 616.

¹⁵ J. Path. & Biochem., 1919, 22, p. 323.

¹⁶ Bioch. Ztschr., 1912, 38, 1501.

¹⁷ Lancet, 1903, 2, p. 446.

¹⁸ Ztschr. f. Immunitätsf., 1911, 8, p. 58.

temperatures. If the action of complement is an enzymelike on, we should not expect changes in temperature to influence noticeably the position of equilibrium between the reacting substances, because the heat change accompanying the reaction is small. However, a much greater alteration with temperature should be shown by the velocity with which the system proceeds toward equilibrium. In most enzyme reactions a rise of temperature of 10 C. doubles or trebles this velocity (Euler¹⁹). But temperature has a twofold influence on enzyme reactions: the one just described and, in addition, an action on the enzyme which becomes more rapidly destroyed or inactivated as the temperature rises. Even at the so-called "optimum temperature" (the position of which depends entirely on the period or phase of the reaction considered) the enzyme undergoes partial destruction during the reaction (Euler). If a comparison is made of the times taken for the reaction to proceed to the extent of one-half, the optimum is apparently lower than if only the first one-fifth of the reaction is considered. The initial velocity will, in general, show no optimum temperature if the time of observation is made short enough.

PROBLEM

The purpose of this investigation was to determine the influences of changes of temperature on the velocity of reaction in hemolysis, and thereby find to what extent hemolysis is similar to true enzyme reactions.

PROCEDURE

Preparation of Hemolytic Amboceptor.—In the preparation of amboceptor, rabbits were injected intraperitoneally with 2 to 3 c.c. of whole defibrinated sheep blood at 5-day intervals until the unit of amboceptor was less than 0.0005 c.c., when 1 c.c. of a 1:20 dilution of guinea-pig serum and 1 c.c. of a 2% suspension of washed sheep red blood corpuscles were incubated for 1 hour at 40 C. in a water bath. The immunized rabbits were bled from the heart by means of a clean all glass aspirating syringe. The blood was transferred to chemically clean test tubes, in which it was allowed to clot while standing on ice over night. The following morning the serum was separated from the clot by centrifugation. After heating in a water bath at 55 C. for 30 minutes, the serum was stored on ice (0-3 C.) until the time of use, when it was removed just long enough in each case to permit the removal of the small amount necessary for the day's tests. The quantity of serum which constituted a unit of amboceptor was found not to have increased during two months' storage.

Preparation of the Complement.—It is a well-known fact that the serums of guinea-pigs vary considerably in complementing power. For this reason, the serums of from 6 to 10 guinea-pigs were mixed so as to equalize the differences in hemolytic activity. Guinea-pigs were bled from the heart, and the serum separated from the clot by centrifugation, after it had stood at room temperature (18 to 23 C.) for $\frac{1}{2}$ to 2 hours. The pooled serum was preserved by the method of Neill,²⁰ in which 0.1 c.c. of a saturated (36%) solution of sodium chloride in distilled water is added to each c.c. of the serum, the mixture then being kept on ice, at a temperature of 0-3 C. It was observed that if the serum was kept in one of the lower compartments of the icebox, at a temperature of 8-10 C., and, if at the time of use, the stock bottle was kept at room tem-

¹⁹ General Chemistry of Enzymes, 1912, p. 231.

²⁰ U. S. Public Health Repts., 1918, p. 1387.

perature for from $\frac{1}{2}$ to 2 hours before being returned to the icebox, that the serum lost over 30% of its complementing activity within 7 days. For that reason the complement was preserved by placing it on the block of ice, where the temperature varied from 0 to 3 C., and, when taken out of the ice compartment for use, the stock bottle was returned with the least possible delay. Guinea-pigs were bled every Monday, so that the serum employed in the tests recorded below was never over 6 days old, and was of undiminished hemolytic potency.

Preparation of Sheep Corpuscles.—The blood suspensions in this work were prepared by washing defibrinated sheep blood by centrifugation. The supernatant liquid which was pipetted off after each washing was replaced by 0.85% sodium chloride solution in distilled water, the packed corpuscles being stirred up carefully after each such replacement by alternately withdrawing and blowing out some of the suspension with a pipet. The final washing of each lot of corpuscles was always continued for the same length of time, at the same speed of centrifugation. Corpuscle suspensions vary considerably when this precaution is not taken, as shown by Kolmer and Brown.²¹ A fresh supply of blood was obtained twice each week, so that in no case was the antigen employed in the tests older than 3 days, although, as Kolmer²² has shown, there is no loss of resistance to hemolysis in 4 days in plain defibrinated blood kept at a temperature below 8 C., while if the packed cells after the third washing are preserved at a low temperature under the salt solution of this washing, they are usually entirely satisfactory for at least 7 to 10 days.

Apparatus for Dilution of Reagents.—The measurement of volumes of 1 c.c. or less of all reagents whether in the preliminary titrations or in the actual tests were made with 1 c.c. "D. R." calibrated pipets, graduated to 0.01 c.c. Dilution of the corpuscle suspension was made in glass stoppered volumetric flasks of 100 c.c. capacity. Dilutions of amboceptor and complement were made in Erlenmeyer flasks, the salt solution being added to the reagents by means of accurately calibrated 5 and 10 c.c. pipets. The pipets were numbered, and in each series of tests the same procedure was followed with the same pipet, so as to add an additional check on the original calibrations.

Tubes for Hemolysis.—In all of this work, Pyrex glass test tubes of $\frac{1}{2}$ x 4-inch size, without lips, were used. All glass apparatus was made chemically clean by first scrubbing with Sapolio, then rinsing several times in hot tap water, immersing in a sulphuric-acid-potassium-bichromate cleaning solution for about half an hour, then rinsing 3 times in tap water, 3 times in distilled water, and finally drying in a hot air sterilizer.

The Thermostat.—The incubations were made in a metal water bath, the temperature of which was controlled within 0.5 C. by means of a gas thermostat.

The Comparator.—The comparator used in comparing colors was a 4 inch cube of soft wood, with vertical and horizontal holes $\frac{1}{2}$ inch in diameter bored through it. This was mounted on a 1 inch block, 5 inches square. The whole apparatus was enameled black on both the inside and the outside. Strips of pure white "milk glass" obtained from an old Beckmann thermometer were used to close the back (distant) end of the horizontal holes. (Most of the "milk glass" now on the market is slightly yellow rather than pure white.) This comparator is similar to that used by Clark and Lubs²³ in colorimetric hydrogen-ion determinations.

²¹ Am. J. Syph., 1919, 3, p. 169.

²² Ibid., 1919, 3, p. 619.

²³ Jour. Bacteriol., 1917, 14, p. 1.

Effect of Various Temperatures on Rate at Which Reaction Proceeds to Equilibrium.—Preliminary titrations were made at 40 C. in a water bath, the incubation time being 60 minutes. A unit of amboceptor was taken as that amount which, in the presence of 1 c.c. of 1:20 guinea-pig complement would exactly cause complete hemolysis of 1 c.c. of a 2% suspension of washed sheep corpuscles in a total volume of 3 c.c., at the foregoing temperature and time. Tests were made at 10, 20, 25, 30, 35, 40, 45, 50, and 55 C. To a series of tubes was added first 1 c.c. (1 unit) of amboceptor and then 1 c.c. of 1:20 guinea-pig complement. The tubes were placed in a water bath until their contents reached the temperature at which the test was to be made. In the 45, 50, and 55 sets, the complement was not warmed to temperatures above 40 C. before its addition to the amboceptor. After the addition of red blood cells (previously warmed), the mixtures were shaken, and immediately replaced in the water bath. These mixtures were incubated for various lengths of time. Therefore, to facilitate the operations, the previously warmed erythrocyte suspension was first added to the tube which was to be incubated the greatest length of time, and then to succeeding tubes of shorter incubation time. This procedure reduced to a minimum the time error which would have resulted had the red blood cells been added to all tubes at "the same time," and, in addition, it decreased the work involved in the later step of centrifugation.

Incubation periods in each series of tests were 1, 2, 5, 10, 15, 20 minutes, and above 20, the difference was 10 minutes, i. e., 30, 40, 50, etc., until the reaction reached its equilibrium. The time was read off on a stop watch. During the entire period of incubation the tubes were frequently shaken so as to prevent agglutination and sedimentation of the cells. By the foregoing procedure one minute after the red blood cells were added to the "one minute" tube all of the tubes were removed from the thermostat, and were placed in previously weighed centrifuge cups. Immediate centrifugation removed all unhemolyzed cells from the "field of action," thus rendering it possible to determine the extent to which the reaction had proceeded in each tube by colorimetrically determining the amount of dissolved hemoglobin in the supernatant liquid. This colorimetric hemoglobin determination was made by comparing the tubes with a set of "standards," in the comparator. Earlier experiments proved to me that if the concentration of the erythrocyte suspension was greater than 2%, it was practically impossible to distinguish between various percentages of hemolysis above certain limits, which decreased as the concentration of the suspensions increased. With a 2% suspension, however, it is possible to read accurately from 0.05% to 100%. The set of standards consisted of:

Tubes	Corpuscles, 2% Suspension	Dist. Water	Complement	= % Hemolysis
1.	0.01 c.c.	1.99 c.c.	1 c.c.	1
2.	0.05	1.95	1	5
3.	0.07	1.93	1	7
4.	0.10	1.90	1	10
5.	0.20	1.80	1	20
6.	0.30	1.70	1	30
7.	0.40	1.60	1	40
8.	0.50	1.50	1	50
9.	0.60	1.40	1	60
10.	0.70	1.30	1	70
11.	0.80	2.20	0	80
12.	0.90	2.10	0	90
13.	1.00	2.00	0	100

The results of a series of experiments in which 1 of the 3 immune rabbit serums was used, are condensed into table 1.

TABLE 1
RESULTS OF EXPERIMENTS IN WHICH ONE OF THREE IMMUNE RABBIT SERUMS WAS USED

Incubation Temp., C.	Minutes																
	1	2	5	10	15	20	30	40	50	60	70	80	90	100	120	140	180
10	0	0	0	0	0	0	0	This set was not carried beyond 30 minutes									
20	0	0	0	2	5	15	20	20	30	35	40	50	60	67	75	85	90
25	0	2	5	7	—	12	22	43	50	70	80	—	90	97			
30	0	2	5	5	5	20	55	70	90	90	95	100					
35	0	5	5	15	20	35	70	80	85	90	95	100					
40	0	5	5	20	50	60	80	90	95	100							
45	5	5	15	50	70	80	90	90	90	90							
50	5	5	10	15	25	35	40	40	40	40							
55	0	5	10	10	10	10	10										

The figures in the table represent hemolysis in percentage (the first horizontal row of numbers represents the length of time of incubation).

Table 2 shows the effect of temperature on the rate of inactivation of complement at the two temperatures at which, from table 1, the inactivation plays the greatest part in the retardation of the reaction. Complement was used in 1 c.c. amounts. A series of tubes were incubated for various lengths of time, chilled in ice water, and then one unit of amboceptor and 1 c.c. of red corpuscle suspension were added to each. All were then incubated for 1 hour at 40 C. in a water bath:

TABLE 2
EFFECT OF TEMPERATURE ON RATE OF INOCULATION OF COMPLEMENT

Incubation Temperature, C.	1 Minute	2 Minutes	5 Minutes	10 Minutes	15 Minutes	20 Minutes
50	60	55	50	50	45	40
55	25	20	15	0	0	0

The percentage hemolysis was read by comparing the test tubes with a set of standards consisting of a series of tubes each containing 1 c.c. of corpuscle suspension and 1 c.c. of amboceptor, but the complement content of which varied from 0.1 to 1.0 c.c., by tenths of a c.c. The total volume in each tube was 3 c.c. This series was incubated for 1 hour at 40 C. in a water bath. After centrifugation, the supernatant liquid was used in the set of standards. Thus it is evident that the "percentage" in this table represents the amount of complement used in each tube, and that the difference between this and 100 represents the amount destroyed in the given time.

From tables 1 and 2 and charts 1 and 2, the following points may be made:

(a) In speaking of the "optimum temperature" for hemolysis, the phase of the reaction must be considered, for the optimum temperature to cause 10% hemolysis is obviously different from that at which 100% hemolysis is most quickly produced. The former temperature is 55 C., while the latter is 40.

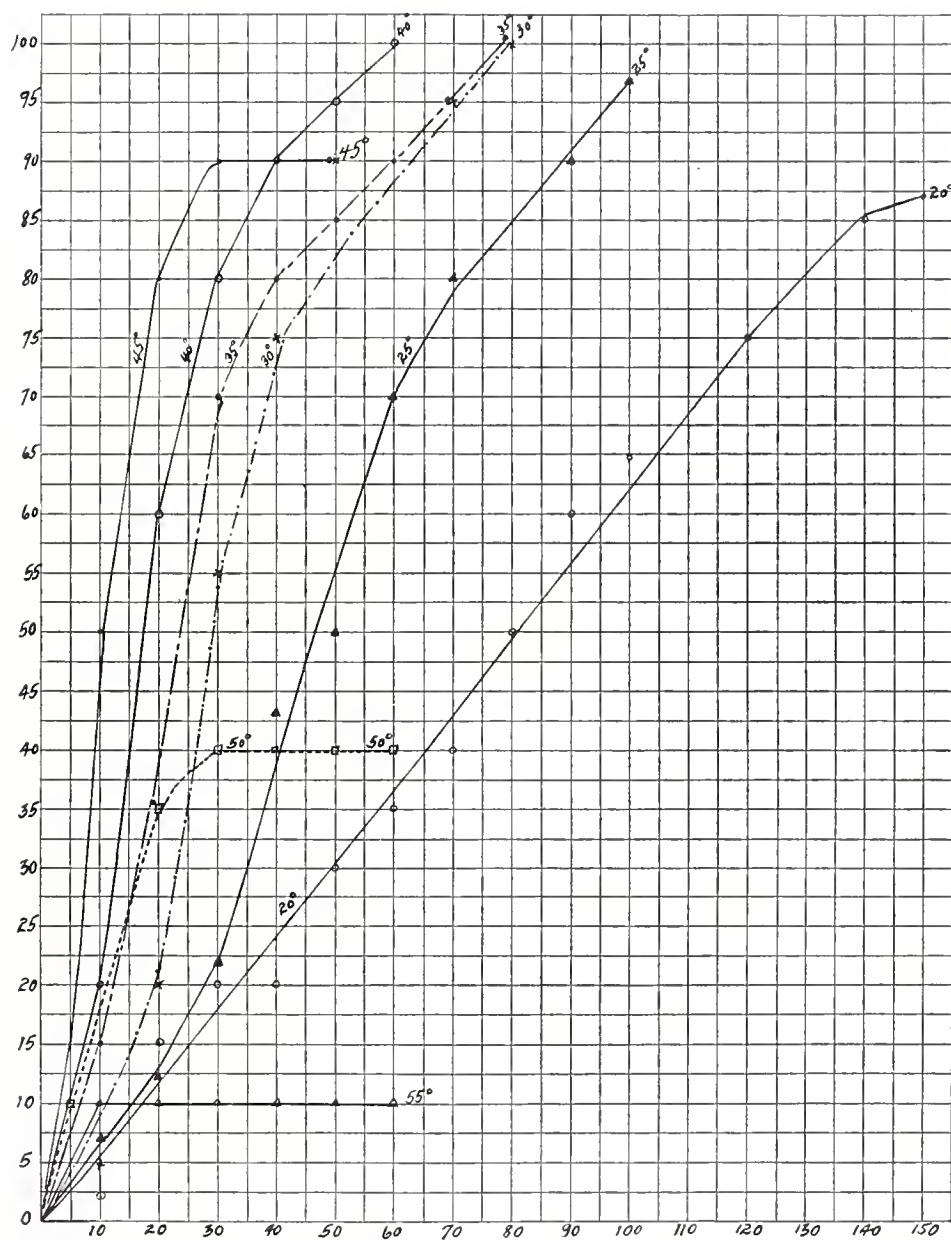


Chart 1.—Effect of temperature on rate at which reaction reaches its point of equilibrium. In Charts 1 and 2 ordinates represent time in minutes, abscissae percentage of hemolysis.

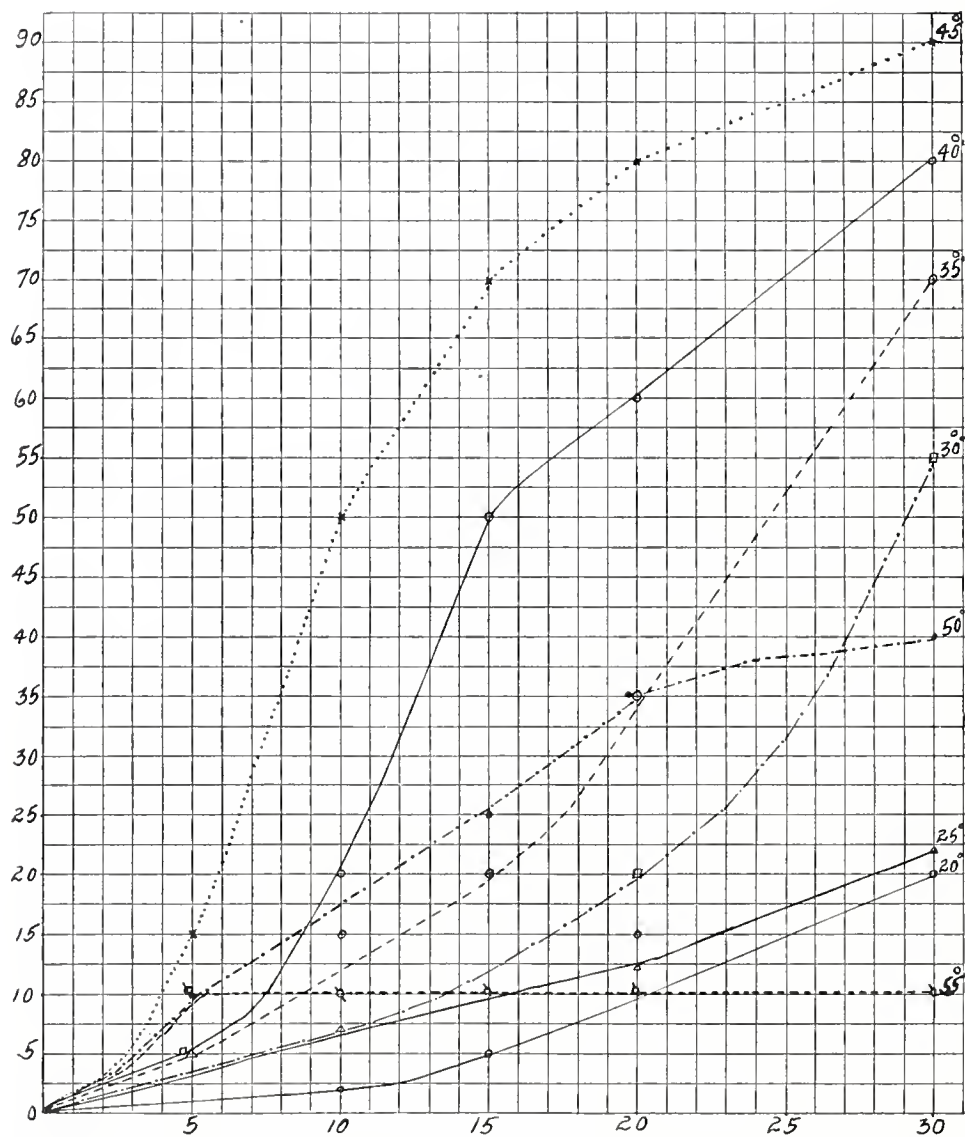


Chart 2.—Effect of temperature on rate at which reaction reaches its point of equilibrium. This chart covers readings which occupy only the first one-sixth of chart 1, and shows the period of "lag" which occurs after mixing of the reagents but preceding lysis.

(b) When only one influence of temperature is considered, namely, its influence on the rate at which the reaction is hastened toward its point of equilibrium, it is seen that the higher temperatures (45-50 and 55 C.) are more effective than are the lower ones.

(c) When, however, the second influence of temperature is considered, namely, its influence in causing the destruction or inactivation of the enzyme-like substance complement, it is at once apparent that the lower the temperature, the less intense is the effect, and the longer is the time which elapses before the effect is noticed. On the contrary, the higher the temperature, the sharper is the break in the reaction curve (chart 1) and the sooner is this effect apparent. An exposure of 5 minutes at 55 C. completely inactivated complement, while at 50 C. the same time of exposure was sufficient to inactivate 50% of it.

(d) A temperature of 40-45 C. is apparently the optimum temperature for hemolysis, for, as Euler¹⁹ points out, "For practical purposes, to know the temperature at which a reaction proceeds most rapidly it is best to consider only times in which the reaction is 90-95% complete."

(e) It is evident that at temperatures below 45 C. there is a comparatively long "lag" period during which the reaction proceeds slowly. Then follows a sudden rise in the reaction curve (chart 2). This rise in the curve is more abrupt for each temperature than for the immediately previous lower temperature, until 50 and 55 C. are reached. Here, due to complement destruction, there is a sharp break toward the horizontal, after the first few minutes.

Is the period of "lag" at the beginning of hemolysis due to a lack of approximation of the reacting substances, or does it follow the "absorption phase" and occur at the beginning of the "reaction, or lytic phase"?

To determine this point, the same technic as that described was followed. The maximum incubation period was 20 minutes. In each case, after making the readings of percentage hemolysis, the supernatant liquid was removed and transferred to another tube. This contained, then, a varying amount of dissolved hemoglobin, any unbound amboceptor and complement and the other soluble substances normally present in the serum which were not carried out of solution by adsorption. That amboceptor, which, during the period of incubation was bound to the red blood cells, was of course, carried out of suspension during the centrifugation. The same is true of any complement which was firmly enough attached to existing amboceptor-corpuscle com-

plexes. Two series of tests were made of this sediment, and one series was made of the supernatant liquid. In one series the sediment at the bottom of the tubes was resuspended in normal salt solution—the total volume of the resulting suspension was 3 c.c. These mixtures were incubated for 1 hour at 40 C. in a water bath. If amboceptor and complement were both absorbed during the preliminary incubation, hemolysis should take place in these mixtures. If, however, one but not the other was absorbed, no hemolysis should take place. In the other series of tests, the sediment at the bottom of the original tubes was resuspended in a little normal salt solution; 1 c.c. of complement was added; the volume was made up to 3 c.c., and the mixtures were incubated in a water bath for 1 hour at 40 C. If amboceptor, but not complement, was absorbed during the first incubation, the addition of complement should result in hemolysis. Table 3 records the results of these experiments:

TABLE 3
RESULTS OF TESTS WITH SEDIMENT

Incubation Temp., C.	Added to Sediment: Saline Solution to Make 3 c.c.						Complement 1 c.c.: Saline Solution to Make 3 c.c.					
	1	2	5	10	15	20	1	2	5	10	15	20
	Min.	Min.	Min.	Min.	Min.	Min.	Min.	Min.	Min.	Min.	Min.	Min.
10	0	0	0	0	0	0	70	90	95	95	95	95
20	0	0	0	0	0	0	90	90	95	95	95	95
30	0	5	10	15	15	15	90	95	95	95	95	90
35	0	5	10	10	10	10	70	80	90	80	70	50
40	0	0	5	10	10	10	80	90	80	70	40	40
45	0	0	0	5	5	5	50	45	45	25	20	15
50	0	0	0	0	0	0	90	85	70	60	20	20
55	0	0	0	0	0	0	90	85	85	85	85	85

The figures give % of hemolysis.

From table 3 it is seen that:

(a) At all temperatures from 10 to 55 C. the amboceptor is almost completely (over 90%) absorbed by the red blood cells during an exposure of only 1 minute. The reason for the incomplete hemolysis in the 35-40-45-50 and 55 C. series to which complement and salt solution were added is that during the preliminary incubation some of the red blood cells were laked, thus decreasing the total number available for lysis in the second incubation.

(b) At no temperature is complement bound to amboceptor-erythrocyte complexes so firmly that it is carried down with the cells during centrifugation. The action of complement on red blood cells is, therefore, probably a surface reaction.

(c) In most cases, all of the amboceptor is absorbed out of the mixtures. This is shown by the fact that there is in most cases no hemolysis when 3 c.c. normal salt solution are added to the sediment after the first incubation. The partial hemolysis (15% was the greatest amount) occurring in some of these tubes was probably due to partial destruction of the cells during the preliminary incubation, this rendering them susceptible to disintegration during the 1 hour incubation which followed. If this partial hemolysis was due to free amboceptor in the mixture, the 1 and 2 minute tubes (left half of table 3) would also have shown hemolysis. Further, since complement is not carried down with the amboceptor-erythrocyte complexes, the addition of red blood cells to the supernatant liquid following the first incubation should show hemolysis if the amboceptor is still free in it. That amboceptor is not free, except during the first minute or two, is shown by the following controls, picked at random from my records:

Addition of $\frac{2}{3}$ c.c. red blood cell suspension to 2 c.c. ($\frac{2}{3}$ of original volume) of the supernatant liquid following the first incubation: Tubes then incubated one hour at 40 C. in a water bath.

	1	2	5	10	15	20 minutes.
10 C.....	15	10	0	0	0	0
30 C.....	5	5	0	0	0	0

(d) The "lag" in hemolysis occurs after the absorption phase has progressed to completion, and therefore occurs in the "reaction, or lytic" phase.

SUMMARY AND CONCLUSIONS

Temperature has a two-fold influence on hemolysis, just as it does on "true" enzyme reactions:

(a) It hastens the reaction to its state of final equilibrium, as in the case of most chemical reactions.

(b) It has a retarding influence which increases as the temperature increases, due to the destruction or inactivation of one of the reacting substances, complement.

The optimum temperature for hemolysis lies between 40 and 45 C., when a reaction running to 90 or 95% completion is considered.

Even at 20 C. the inactivating effect of long continued heating on complement is apparent. At higher temperatures this effect becomes more noticeable, as shown by the earlier and sharper breaks in the reaction curves—(chart 1).

Absorption of amboceptor by red blood cells is more than 90% complete during the first minute, even at 10 C. Increase in temperature therefore has but little effect on this phase of the reaction.

The "lag" which occurs in hemolysis immediately after the mixing of the reagents, takes place in the lytic phase and not in the absorption phase.

Complement is not firmly bound to amboceptor-erythrocyte complexes, and centrifugation of the mixtures leaves it in suspension.

The action of complement on amboceptor-erythrocyte complexes is probably a surface phenomenon.

Complement is completely inactivated in less than 10 minutes at 55 C. This inactivation is over 75% complete in the first minute. At 50 C., the inactivation is over 50% complete in the first 5 minutes.

STUDIES ON THE THERMAL DEATH TIME OF SPORES OF CLOSTRIDIUM BOTULINUM

2. THE DIFFERENTIAL STAINING OF LIVING AND DEAD SPORES *

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It is well known that with certain types of cells, such as human leukocytes, it is possible by a special method of staining to distinguish living from dead cells. In such cases the dye penetrates the dead cell more readily than the living.

We have no knowledge of a similar method to distinguish living from dead bacteria, although silver nitrate has a selective action for dead bacteria. The criterion commonly used to determine viability of bacteria has been the ability to grow in cultures. This is not a direct method, since it requires a good deal of time and involves many factors not inherent in the cell itself, such as optimum germination and growth conditions, which are sometimes difficult to control. A staining technic by which living and dead bacterial cells could be quickly differentiated would obviously be of great advantage in many types of work.

This paper describes a difference in dye penetration between individual spores of *Cl. botulinum* and presents evidence in favor of the view that penetration of the dye into the spore is coupled with loss of viability and death of the spore. This affords a quick method of determining approximately the number of dead spores in a given culture.

Spores of *Cl. botulinum* can be divided into two groups, depending on the extent of the penetration of carbol-fuchsin. One group consists of spores into which the carbol-fuchsin penetrates only a short distance. These are called "ring forms" because of the narrow border of fuchsin surrounding the unstained center. The other group consists of spores into which the carbol-fuchsin penetrates to the center of the cell and which are called "solid staining forms." Between the two extremes are a relatively small number of intermediate forms which represent all stages of dye penetration and which are divided on the basis of the appearance of the center of the spore, those with distinctly white centers being classed as ring forms and those with pinkish centers as solid staining forms. This is an arbitrary line of division which was

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* These experiments are a part of an investigation of botulism made in California by the U. S. Public Health Service, Leland Stanford Junior University and the University of California, under a grant from the National Cannery Association, The Cannery League of California and the California Olive Association.

chosen because it seemed to be more clearly defined than any other and, therefore, likely to give more consistent differential counts.

The penetration of the dye appears to bear no definite relation to the age of the spore, since both ring and solid forms are found in the subterminal spore stage and in the supposedly more mature free spore stage; neither does it seem to be dependent on the age of the culture, since 3 cultures of strain 58, varying in age from 2 weeks to 7 months, were found to contain practically the same percentages of ring and solid form.

There is a definite relationship, however, between the penetration of carbol-fuchsin and the death of the spore by heat, as indicated by the fact that smears of unheated, viable spore cultures, when stained, contain a large majority of ring forms, whereas cultures sterilized by heat contain solid staining spores only.

EXPERIMENTS TO DETERMINE THE RELATION OF HEAT AND SUBSEQUENT LOSS OF VIABILITY TO THE DIFFERENTIAL STAINING OF SPORES OF *CL. BOTULINUM*.

A glucose broth culture of *Cl. botulinum*, Stanford strain 58, provided spores for the following experiments. The culture contained approximately 100 million spores per c.c.

Exper. 1.—To determine the effect of the heat of fixation on the percentage of solid staining spores.

Smears from the stock culture were made on 2 slides and thoroughly air dried. The smear on one slide was then fixed in the usual manner by being passed 3 times through the flame of a Bunsen burner. The smear on the other slide was not fixed by heat. The slides were stained at the same time in steaming carbol-fuchsin, destained with acetone and counterstained with methylene blue.

The following staining technic is used:

1. Immerse slides for 2 minutes in a beaker of steaming carbol-fuchsin (basic-fuchsin, Grubler, 1.5 gm. dissolved in 15 c.c. of 95% alcohol are added to 5 c.c. of melted phenol crystals in 85 c.c. of distilled water).
2. Wash with distilled water immediately after removal from the stain, and blot dry.
3. Destain with absolute acetone, the slide being flooded 2 or 3 times and the acetone poured off almost at once.
4. Wash with water before the acetone evaporates to dryness; and blot dry.
5. Counterstain with Loeffler's methylene blue for 2 minutes.

The period of immersion in the carbol-fuchsin varies slightly with the strain of the organism used. The shortest time that will stain the ring forms gives the best red and blue differentiation.

A differential count of 1,000 spores was made on each smear, to determine the percentages of ring and solid staining spores. The unheated smear contained 33 solid forms per 1,000 spores and the heated smear 186 per 1,000, showing that the heat of fixation altered about 15% of the spores so that they became more permeable to carbol-fuchsin.

Since it is not possible to control accurately the amount of heat applied to the spores when slides are fixed by passage through a flame, this procedure

was abandoned. In the succeeding experiments the films were thoroughly air dried and stained without heat fixation.

Exper. 2.—To determine the effect of the heat in steaming carbol-fuchsin on the percentage of solid staining.

Four air dried smears of the stock culture were treated as follows: One was placed in cold carbol-fuchsin for 48 hours; the second, the third and fourth slides were placed in steaming carbol-fuchsin (under 100 C.) for 1, 2 and 3 minutes, respectively. They were then destained and counterstained as in *exper. 1*. Differential counts of 1,000 spores each were made, and approximately the same percentage of solid staining forms were found in each smear. The unheated spores of another broth culture of strain 58 were stained in cold carbol-fuchsin for 10 minutes, then destained and counterstained. The result

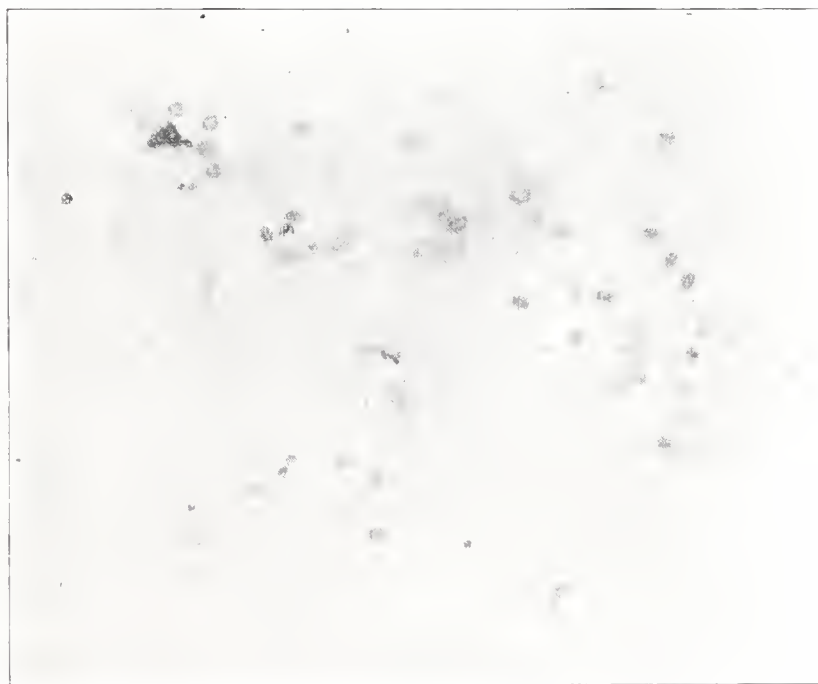


Fig. 1.—Spores of *Cl. botulinum*, stained with carbol-fuchsin, destained with acetone and counter-stained with methylene blue, showing ring and solid types of staining.

was the same, a few solid staining forms and a great majority of ring forms. Thus the technic of staining in steaming carbol-fuchsin (under 100 C.) for from 1 to 3 minutes has little or no effect on the percentage of solid staining spores. The 18 air dried slides of unheated spores mentioned in these experiments contained less than 3% of solid staining spores each.

Exper. 3.—To determine the effect of a given exposure to heat on the staining reaction of spores in duplicate tubes.

Two c.c. of the stock broth culture were placed in each of fourteen small tubes. The tubes were sealed with an oxygen flame about one inch above the surface of the liquid. Seven tubes were placed in each of 2 racks and the racks lowered into hot oil. The initial temperature of the oil bath was such that both the contents of the tubes and the oil became stabilized at 115 C. within 3 minutes. One rack was removed at the end of 4 minutes and the second

rack at the end of 8 minutes. Thus the 7 tubes in each rack were heated for exactly the same length of time.

The tubes were chilled immediately in cold water and a smear was made of the contents of each tube. Three or 4 smears were placed on each slide together with a smear of unheated spores from the stock culture, which served as a control of the staining technic. The slides were air dried and stained as in exper. 1. and a differential count of 1,000 spores was made on each smear.

The control smears of unheated spores on the 4 slides contained from 1.2% to 3.0% of solid staining spores, the average being 2.1%.

TABLE 1

DIFFERENTIAL COUNTS OF UHEATED AND HEATED SPORES OF CL. BOTULINUM, STRAIN 58, SHOWING THE CHANGE IN THE PROPORTIONS OF RING AND SOLID FORMS CAUSED BY HEATING FOR DIFFERENT LENGTHS OF TIME AT DIFFERENT TEMPERATURES IN A BROTH MEDIUM

Minutes Heated	Percentage in 1,000 Spores							
	Heated 100 C.		Heated 107.5 C.		Heated 115.5 C.		Heated 121 C.	
	Ring	Solid	Ring	Solid	Ring	Solid	Ring	Solid
1.....	9.07	9.3	93.5	6.5
2.....	83.9	16.2	83.0	17.0
4.....	6.1	93.9
5.....	95.5	4.5	77.6	22.4	26.0	74.0	0.2	99.8
6.....	0.2	99.8
7.....	0.0	100.0
8.....	0.1	99.9
10.....	89.1	10.9	63.1	36.9	6.2	93.8	0.1	99.9
11.....	6.8	99.2	0.1 ?	99.9
15.....	85.1	14.9	51.9	48.1
20.....	45.7	54.3	0.0	100.0
30.....	79.1	20.9	20.9	79.1	0.1 ?	99.9
40.....	6.1	93.9	0.1 ?	99.9
45.....	72.2	27.8
50.....	0.8	99.2
60.....	38.3	61.7	0.2	99.8
70.....	0.0	100.0
120.....	12.5	87.5
180.....	4.5	95.5
270.....	0.5	99.5
Controls of unheated spores	98.9 99.1 99.2	1.1 0.9 0.8	98.1 98.4	1.9 1.6	98.1 98.3	1.9 1.7	98.8 98.5	1.2 1.5

The smears of the 7 tubes heated at 115 C. for 4 minutes contained from 77% to 83.5% solid staining spores, with an average of 80.5%.

The smears of the 8-minute series contained from 95.5% to 97.2% solid staining spores with an average of 96.3%.

The counts of the control smears of unheated spores agree so closely as to prove that the staining was practically uniform and that smears of the unheated culture contain a small percentage of solid staining spores.

The experiment demonstrates:

1. That heat increases the permeability of the spores to carbol-fuchsin;
2. That individual spores vary in the amount of heat necessary to render them completely permeable, hence the higher average percentage of solid staining forms in the 8-minute group than in the 4-minute group;
3. That for a given culture the percentage of spores, completely penetrated by the dye, bears a definite relation to the amount of heat as indicated by the close agreement of counts of duplicate tubes. Compare also exper. 4, table 1, 115.5 C. for four and eight minutes.

It is evident, however, that there is a wider range of variation between the highest and lowest counts of the series heated for 4 minutes than of that heated for 8 minutes. The variation is relatively small in either case and might well be attributed to reasonable variation in technic, except for the fact that comparative results of other series of spore counts, not recorded in this paper, indicate that if the amount of heat exposure is still less the range of variation is wider than that of the four minute series. Apparently the range of variation between counts of duplicate heated samples becomes proportionately less as the thermal death point is approached, due quite possibly to the relative decrease in the number of ring forms which remain to be penetrated by dye. It is possible that a larger differential count on each slide (10,000 instead of 1,000) would produce more uniform results.

Exper. 4.—To determine the effect of the degree of temperature and length of exposure on the staining reaction of the spores.

Four series of small tubes were prepared as in *Exper. 3*, and heated in hot oil at 100, 107.5, 115.5 and 121.1 C. respectively. The heating was done in the same manner as in *exper. 3*, except that only one tube was removed from the bath at each time interval.

The tubes were chilled immediately and smears made of the contents. A control smear of the unheated spores was placed on each slide. The slides were air dried and stained as in *exper. 1*. A differential count of 1,000 spores was made on each smear. The percentages of ring and solid forms are given in table 1.

The counts of the nine control smears of unheated spores varied from 0.8% to 1.9% solid staining forms, showing that the staining technic was practically uniform for the 9 slides and that the percentage of solid staining spores in the unheated culture was extremely small.

The counts of heated spores show:

1. That the percentage of solid staining spores (spores which have been rendered permeable by the heat) increases regularly and in direct proportion to the duration of heat exposure.

2. That the change in permeability takes place more rapidly at higher than at lower temperatures.

The percentages of "intermediate" forms (spores partially penetrated by the dye) is relatively small in the smears suggesting that the change in permeability of the individual spore takes place comparatively quickly.

These observations have been confirmed by a number of other series of counts on strain 58 and also on other strains both of type A and type B. The results are remarkably consistent.

SUMMARY

The facts brought out in these experiments prove that heat increases the permeability of the spores of *Cl. botulinum* to carbol-fuchsin and that the amount of heat necessary to bring about the change varies with the individual spores. In series of cultures heated for varying lengths of time, the increase in solid staining forms parallels the curve of loss of viability or death of the spores, as indicated in cultural tests. The thermal death time of the species (which is in reality the thermal death time of the most heat resistant spore tested) approximates closely the point in the heating process at which all the spores have become

dye permeable (100% solid staining). Comparison of the maximum survival for spores heated in broth (table 2) with the corresponding percentages of solid staining forms in smears of spores heated in the same medium (table 1) suggests that loss of viability slightly precedes complete dye penetration (solid staining).

Solid staining forms in smears quite definitely represent dead spores, since 100% of the spores in known sterilized cultures stain in this way and none stains as ring forms. Viable spores, on the other hand, are represented by "thin-walled" ring forms which constitute a great majority in smears of unheated cultures. The actual point of loss of viability is probably represented by some stage in partial dye penetration, which apparently does not coincide exactly with the arbitrary line of division adopted in differential counting, although there appears to be a consistent relation between the two (compare tables 1 and 2).

TABLE 2
MAXIMUM SURVIVAL IN MINUTES OF SPORES OF *CL. BOTULINUM* WHEN HEATED AT
DIFFERENT TEMPERATURES IN DIFFERENT SUBSTRATA ¹

Substrata	100 C.	107.5 C.	115.5 C.	121.1 C.
Broth.....	225	40	7	4
Brain.....	270	45	10	6
Oil-stratified broth.....	315	80	42	22

Exper. 4 has been repeated with 4 other strains of *Cl. botulinum*, including type B as well as type A, and the results are entirely similar to those already described for strain 58.

Spores of viable and of heat-sterilized cultures of *B. anthrax*, *B. subtilis*, *B. vulgatus* and *B. cereus-fluorescens*, when stained by this method, give the same results as *Cl. botulinum*.

The effect of disinfectants on the staining reaction of spores has not been determined and is reserved for a later study.

The method of determining the thermal death point of the spore by its change in dye permeability not only agrees fairly closely with cultural tests, but its results appear to be, if anything, rather more consistent. It cannot, obviously, replace the cultural method. Nevertheless, the author believes that in heat resistance experiments much time and labor can be saved if preliminary tests are made by this method to outline the scope of the cultural tests and serve as a control on the cultural technic.

¹ Studies on Thermal Death Time of Spores of *Cl. Botulinum*, Dickson, Burke, Beck, Johnston, King, Jour. Am. Med. Assn., 1922, 79, p. 1239.

HYDROGEN-ION STUDIES

VII. HYDROGEN-ION CONCENTRATION RANGE OF PRECIPITIN REACTION (SHEEP SERUM)

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The precipitin reaction made by bringing together in solution an antigen and its homologous immune serum chemically is regarded as the mutual precipitation of one colloid by another. During the last few years the importance of the hydrogen-ion concentration of the medium on the chemical behavior of the contained colloids has been demonstrated. Colloidal protein solutions generally are considered solutions of amphoteric electrolytes, and the colloidal particles in an alkaline medium (in reference to the iso-electric point of the colloid) carry a negative electrical charge, and in an acid medium, a positive charge. At a hydrogen-ion concentration specific for the protein there is no difference in the electrical potential between the particles, and the medium and the particles seem to be without an electrical charge. This is called the iso-electric point of the protein. Loeb¹ has demonstrated that it marks the turning point of the chemical change which determines the nature of ionization and that the ionization determines the electrical charge. Ionized as an acid and combined with a cation on the alkaline side of the iso-electric point, the protein reacts as an anion and carries a negative electrical charge; ionized as a base and combined with an anion at reactions acid to the iso-electric point, the protein carries a positive charge. Hardy's rule states that colloids in solution carrying an electrical charge are precipitated by ions of opposite electrical charge. Colloids have been divided into hydrophilic and hydrophobic, according to the ease with which their solutions or suspensions are precipitated by electrolytes. Hydrophilic colloids (sodium caseinate), according to Loeb² require high concentrations of electrolytes for precipitation, while hydrophobic colloids (casein chloride) require low concentrations. In the hydrophobic group, the precipitating ion of the salt has an electrical charge opposite to that of the colloid particle (Hardy's rule), while no such relationship exists in the precipitation of colloids of the hydro-

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¹ Proteins and the Theory of Protein Behavior, 1922.

² Jour. Gen. Physiol., 1922, 4, p. 187.

philic group. Loeb regards the precipitation of the hydrophobic colloids as due to the diminution or annihilation of the osmotic pressure and the potential difference between the surfaces of a micellum and the surrounding solution (forces concerned with the Donnan equilibrium). Loeb offers no explanation for precipitation in the hydrophilic group.

Regarding the physical phenomenon of the precipitation of one colloid by another when mixed together quickly and uniformly, Biltz³ suggests the following rules:

If to a given colloidal solution, one of the opposite sign is added in small proportion, there is no precipitation. As the quantity of the second increases, the coagulative action follows parallel until a proportion is reached which causes immediate coagulation. As the amount is still further increased, coagulation ceases; that is, there is an optimum precipitation for certain proportions, and when these favorable proportions are exceeded on either side, no precipitation occurs.

The influence of the hydrogen-ion concentration on the agglutination of bacteria by homologous immune serum has been observed by Krumweide and Pratt,⁴ by Michaelis and Davidsohn,⁵ and recently by Northrop and DeKruif.⁶ All find the amount of immune serum required to agglutinate is a minimum near the iso-electric point of the organism.

There seems to be no mention of similar studies with the precipitin test, although by analogy a like relationship may be expected to obtain as regards precipitation near the iso-electric points of the antigenic protein substances contained. Reports of studies with the agglutination of bacteria by immune serum mention the difficulty of determining the smallest trace of agglutination in the various dilutions. With the precipitin test where clear solutions of antigen and antiserum may be prepared, the slightest turbidity on mixing can be recognized. Anti-sheep immune rabbit serum was prepared by injecting rabbits repeatedly with sheep serum. After trial experiments, dilutions of immune serum and sheep serum were prepared with increasing concentrations of alkali (NaOH) as follows:

N/10 NaOH c c.....	0.025	0.05	0.075
Distilled water.....	0.975	0.95	0.925
Immune serum, 1: 1 in water.....	0.5	0.5	0.5
Sheep serum, 1: 1 in water.....	0.5	0.5	0.5

³ Ber. d. deutsch. Chem. Gesellsch., 1904, 37, p. 1095.

⁴ Ztschr. f. Immunitätsf., 1903, 16, p. 517.

⁵ Biochem. Ztschr., 1912, 47, p. 59.

⁶ Jour. Gen. Physiol., 1922, 4, p. 655.

The tubes stood in the icebox over night, and then the tube with the faintest trace of precipitation was noted. The hydrogen-ion concentration of the liquid in this tube was determined according to the gas chain method at 25 C. In repeated experiments, the faintest reaction was observed at the hydrogen-ion concentration expressed by P_H 9.4.

After trial experiments with the acid range of precipitation, similar dilutions were prepared with N/100 HNO_3 . As the hydrogen-ion concentration approached P_H 6, flocculation became greater and reached a maximum at that point. However, the addition N/100 HNO_3 up to that concentration causes in sheep serum a precipitate without immune serum, and demonstrates that a protein substance in sheep serum is precipitated by acid at P_H 6, its iso-electric point. Such acid precipitation of a protein in solution at its iso-electric point is generally known, particularly through the work of Michaelis.⁷ Having observed this relationship with the precipitin test, namely, an increasingly large flocculation up to the iso-electric point of a protein substance in the sheep serum, iso-electric protein was prepared by adding a definite amount of N/100 HNO_3 to a volume of sheep serum, allowing the flocculi to settle out, centrifuging at high speed, pouring off the supernatant liquid, and washing (3 to 4 times) with distilled water. This washed white precipitate was then dissolved in distilled water alkalized with a little N/100 NaOH. Chemically this protein belongs to the serum globulin fraction of the sheep serum, for it is precipitated from its alkaline solution by half saturation with ammonium sulphate. Repeated tests with protein solution prepared in this way from sheep serum demonstrated its iso-electric point to be P_H 6. Precipitin tests with the alkaline solution of this protein reacted with immune serum again up to a reaction of P_H 9.4.

The objection may be made that adding N/10 NaOH in amounts sufficient to inhibit the precipitin reaction destroys or alters profoundly the protein substances concerned. In order to test this, enough N/100 HNO_3 was added to neutralize the alkali present in tubes in the range where precipitation was inhibited, and although having stood for 18 hours in contact with alkali concentrated enough to prevent precipitation, neutralization of the excess alkali by acid permitted precipitation to occur in the usual way.

⁷ Praktikum der Physikalischen Chemie, 1921.

The supernatant liquid of the sheep serum, after precipitation of a protein substance by acid, when neutralized by adding a small amount of N/10 NaOH gives precipitin tests in fairly high dilutions indicating that not all of the antigenic substance is precipitated, or more likely, that more than one antigenic protein is concerned in the precipitin reaction.

DISCUSSION

In the mutual precipitation of one colloid by another, a colloid of one electrical charge is precipitated by a colloid of an opposite charge, and the precipitation so accomplished is dependent on or associated with the neutralization of their electrical charges. It is probable, then, that oppositely charged colloids are brought together in solution when antigen is mixed with its homologous immune serum. The ionization and sign of the charge on the colloids, according to Loeb,¹ are dependent on the hydrogen-ion concentration, and when two colloids of opposite charge exist in a medium of a certain reaction, the one with a negative electrical charge is on the alkaline side of its iso-electric point, while the one with the positive charge is on the acid side of its iso-electric point.

Certain colloids at their iso-electric point separate out in flocculi. In sheep serum, a certain protein fraction manifests the flocculation phenomenon, and its iso-electric point can be determined closely after acid precipitation, washing with distilled water and then redissolving with a little alkali. This protein substance is at least one of the proteins concerned with precipitation by immune serum, and with it as antigen alone immune serum forms a precipitate in a hydrogen-ion concentration up to that (P_H 9.4) marking the limit of precipitation in mixtures of native sheep serum and homologous immune serum. It is apparent that the protein substance precipitated from sheep serum by the addition of acid up to a certain hydrogen-ion concentration is on the alkaline side of its iso-electric point in the native sheep serum. The immune substance concerned with the precipitation of this particular protein fraction reacts up to P_H 9.4 which suggests that at this point its chemical activity changes and its electrical charge becomes reversed. That the iso-electric point of the immune substance is as alkaline in reaction as P_H 9.4 is a view not altogether in harmony with ideas already expressed. DeKruif and Northrop⁸ suggest P_H 6 or 7 as the place where the antibody becomes negatively charged. However this may be, it is interesting that there

⁸ Jour. Gen. Physiol., 1922, 5, p. 127.

is a difference of P_H 1.7 between the iso-electric point of the acid-precipitated protein substance in serum and P_H 7.7, approximately the reaction of blood serum after the escape of carbon dioxide; and that there is a similar difference between P_H 7.7 and P_H 9.4, the reaction where precipitation ceases. It may be that the iso-electric point of other antigenic proteins will manifest a similar relationship, the precipitation with immune serum occurring up to a reaction as alkaline as the iso-electric point of the antigen is acid to the reaction of native blood serum.

SUMMARY

The hydrogen-ion concentration range of mixtures of sheep serum and homologous immune rabbit serum lies between P_H 6 and P_H 9.4.

Maximum precipitation occurs near the acid end of the range, that is, near the iso-electric point of a protein fraction precipitating from sheep serum at its iso-electric point.

Sheep serum and homologous immune rabbit serum mixtures in a medium too alkaline for precipitation will so react when the excess alkali has been neutralized by a small amount of acid.

A BACTERIOLOGIC STUDY OF VULVOVAGINITIS OF CHILDREN

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Vulvovaginitis in girls of school age leads to exclusion from school and to economic waste on other accounts. The treatment of the condition in the homes of the poor and in the dispensary is unsatisfactory and prolonged. In girls of pre-school age it necessitates frequent visits to the dispensary, with a consequent loss of time for the mother or other adult member of the family who must accompany the child, before the latter can be considered free of infection and no longer a source of danger to others. If the cases are hospitalized, the economic loss is again great.

Isolation and exclusion from school are based on the belief that the infection is gonorrheal. For some of the institutional epidemics the specific character of the process has been established, but that all patients with vulvovaginitis reporting to dispensaries for treatment are of similar etiology appeared to require further investigation. There may be some question as to the necessity for isolation and exclusion from school of cases not due to the gonococcus, if such cases occur. Nonspecific cases also may possibly require a different method of treatment, or may yield more readily to the same kind of treatment, which is found most efficacious against the gonorrheal variety.

PLAN OF INVESTIGATION

To study these and other points, all children up to 15 years of age reporting to the Dispensary of the Michael Reese Hospital with discharge from or inflammation about the vulva were referred to a single clinic conducted by one of us (Stein). Smears and cultures were made at the time of the first visit; at two weeks' intervals, on subsequent visits, smears and cultures were made to determine the effects of treatment. A uniform method of treatment was carried out; this and the results of the method in the cases studied have been previously reported

(Stein¹). Every case in which the smears showed the presence of leukocytes with intracellular, gram-negative diplococci of characteristic gonococcus morphology was tentatively considered specific in character. The further attempt was made to isolate the gram-negative organism in these cases and to determine by cultural and immunologic reactions whether it actually was the gonococcus. If the smear contained free gram-negative diplococci, the case was considered suspicious or possibly gonorrheal, and further search was made at later examinations for intracellular organisms; these cases were also studied culturally to determine whether the extracellular gram-negative cocci were or were not gonococci. Cases in which the smears contained no gram-negative diplococci on repeated examination were considered nongonorrheal; cultures were made from such cases to determine the nature of the nongonorrheal flora and its possible relation to the process present, and also to learn whether the gonococcus could be cultivated from cases in which gram-negative cocci were never seen in smears.

The smears and the primary cultures were made at the dispensary and conveyed to the laboratories of the Nelson Morris Institute, where the bacteriologic work was carried out. The cultures were made on reduced oxygen plates by surface streaking, the mediums and methods used being described further on. The plates were placed in a small, portable fireless cooker, kept at approximately 38 C. by means of a heated soapstone; the same contrivance was used for transporting the plates to the dispensary, so that they were kept warm from the time they left the laboratory until they were returned and placed in the incubator.

DIRECT SMEAR EXAMINATIONS

Smears and cultures were taken in 66 cases. Twenty-four of these cases were discarded because only one negative smear or one negative or contaminated culture was examined. The smears were stained by the standard gram technic, carefully studied, and the kinds of bacteria present recorded. Of the 42 cases subjected to more detailed bacteriologic study, 15 were considered positive as determined by smear or cultural examination; this number includes one case in which a smear was not examined, but a positive culture was obtained. In the total of 14 cases with positive smear findings, the number of smear examinations varied from 1 per case to as many as 10. In 2 cases in which smears were examined only once, in 1 case which was examined twice, and in 1 case with 8 repeated examinations, only positive smears were obtained. In each of the remaining 10 positive cases, from 4 to 12 successive examinations were made, a total of 78 in all, of which 33 showed the presence of the gonococcus.

¹ Surg., Gynec. & Obst., 1923, 36, p. 43.

In 33 of the 47 positive smears, the gonococcus was the only organism seen. In the 14 remaining positive smears, the gonococcus was associated with other organisms: 12 times with gram-positive diplococci, once with gram-positive diplococci and gram-positive bacilli, and once with gram-positive and gram-negative bacilli.

In 45 negative smears from the 15 positive cases and in 43 smears from 27 cases in which the gonococcus was never detected, either by smear or cultural examination, the bacterial flora was more or less variegated. Gram-positive diplococci occurred in 85 of the total 88 negative smears, 28 times alone and 17 times associated with gram-positive bacilli of the diphtheroid type. In 14 smears, gram-positive diplococci and gram-positive bacilli were associated with other organisms, 11 times with gram-negative bacilli, twice with gram-negative cocci not gonococci, and once with gram-negative cocci not gonococci and with gram-negative bacilli. Combinations of gram-positive diplococci in the absence of gram-positive bacilli were as follows: 18 times with gram-negative bacilli alone; 5 times with gram-negative diplococci not gonococci; once with gram-negative cocci not gonococci and with gram-negative bacilli; once with the Döderlein bacillus; and once with the Döderlein bacillus and with gram-negative bacilli. Two smears contained gram-positive bacilli of diphtheroid type alone and 1 contained Döderlein bacilli alone.

In a total of 135 carefully examined smears from 15 positive and 27 negative cases, the relative order of frequency of the organisms encountered, either alone or in combination, was as follows: gram-positive diplococci, 98; gonococcus, 44; gram-positive bacilli, 35; gram-negative bacilli, 33; gram-negative diplococci not gonococci, 9; Döderlein bacilli, 3.

There was an absence of organisms in 17 smears; but in all except 5 of these one or more types of bacteria were obtained culturally. Two of the 5 cultures were contaminated, leaving just 3 cases which were free of all bacteria, both on direct and cultural examination. Cultures frequently gave evidence of other organisms in addition to those seen on the smear, and occasionally an entirely different type.

In addition to the 42 juvenile cases studied culturally, smears and cultures were made from 6 adult female cases, as controls of the methods applied to the juvenile cases. All of the adult cases yielded the gonococcus on both direct and cultural examination; in 2 of these, the organism was obtained from fallopian tubes removed at operation.

CULTURAL STUDIES

The primary aim of the cultural study was the isolation of the gonococcus, for the purpose of determining the reliability of the direct smear method of diagnosis. However, the great frequency with which gram-positive diplococci and bacilli and gram-negative bacilli were encountered necessitated cultural study of these organisms also for the purpose of determining their nature and their possible relation to vulvar and vaginal inflammatory processes in which the gonococcus could not be detected.

GNOCOCCUS

Before attempting to isolate the bacteria from suspicious gonorrheal cases, different methods of cultivation were tested on 2 gonococcus strains kindly furnished by Dr. R. D. Herrold. These methods have recently been thoroughly

reviewed by Erickson and Albert,² and only the methods used in the actual cultural work will be described. These were as follows:

The base of the medium was nutrient bacterial agar of P_H 7.6 reaction, in which dibasic sodium phosphate was substituted for sodium chloride. Bottles containing 70 c.c. of this medium were boiled to melt the agar and were then transferred to a waterbath at 56 C. When the melted agar was cooled to this temperature, 5 c.c. of defibrinated human blood and 25 c.c. of ascitic fluid were added. The agar was then poured into tubes, the tubes being sloped and incubated over night to insure sterility. They were then stored in the refrigerator until used. Plates on which the original cultures were taken were made of the same medium, which was freshly mixed and poured on the day used.

The primary plate cultures were grown under reduced oxygen tension, the advantage of this method for the cultivation of the gonococcus having been described by Wherry and Oliver.³ The exact method used by us is the one devised by Herrold,⁴ who applied the reduced oxygen tension technic to plate cultures. This simplifies isolating the gonococcus when it is mixed with other bacteria. Two agar plates are used, one being inoculated with the loop from the discharge, the other streaked with *B. subtilis*. They are placed together with the open sides facing each other, and a closed rubber band $\frac{3}{4}$ to 1 inch wide encircles both, enclosing the edges. Satisfactory bands were obtained by cutting discarded rubber gloves into pieces of the required width. Typical gonococcus colonies were picked from these plates on to culture tubes of the same ascitic blood agar, and the oxygen tension was reduced by passing the mouth of the inoculated tube several times through the flame, and maintaining this diminished oxygen tension by tightly closing the tube with a rubber stopper, according to the method of Swartz.⁵

As a base for our fermentation tests, we have employed a sugar-free broth plus ascitic fluid. Veal infusion broth was made sugar-free by planting with *B. coli* and incubating for 24 hours. To the filtrate were added 1.5% peptone and 0.5% dibasic sodium phosphate. After adjusting the reaction to P_H 7.6 and tinting with azolitmin, the medium was tubed in 4 c.c. amounts and autoclaved. Once c.c. of ascitic fluid was then added to each tube; 0.5 c.c. of a 10% solution of the sugar in distilled water was added to each tube of the ascitic broth. The tubes were then incubated over night to determine their sterility.

The inoculated plates were incubated at 37.5 C. for 48 hours and were then examined. The various types of colonies were identified by their gross appearance and by stained smears. Selected colonies were transferred to suitable mediums for growth. In attempting to isolate gonococci in primary cultures and subcultures, it is essential that the medium be warmed at 37.5 C. before inoculation, and thereafter maintained at that temperature.

On the ascitic blood-agar medium, gonococcus colonies in 48 hours are usually discrete, and they are slightly elevated above the surface of the medium, having a central area slightly more elevated than the margins. The surface is glistening and light gray, and the colonies when viewed by transmitted light are transparent. To the unaided eye, the colonies appear to be circular in outline, but with a lens the edge is scalloped, and often radial striations are noted. After some days, small opaque patches of supergrowth

² Jour. Infect. Dis., 1922, 30, p. 288.

³ Ibid., 1916, 19, p. 288.

⁴ Jour. Am. Med. Assn., 1920, 74, p. 1716.

⁵ Jour. Urol., 1920, 4, p. 325.

develop; this has also been observed by Blair Martin,⁶ Hermanies,⁷ and Tullack.⁸

In the identification of gram-negative cocci, agglutination tests with known antigenococcus serum and fermentation tests in sugar broths were used. The sugars used were levulose, galactose, maltose and dextrose. We are indebted to Dr. R. D. Herrold for the polyvalent immune serum which was used in the diagnostic agglutination tests. All of the gram-negative cocci agglutinated by this antigenococcus serum reacted typically on the sugars used.

Of the 42 cases studied, 15, or 35.7%, gave positive gonococcus smears. Eight, or 19.0%, of the total gave positive gonococcus cultures; 53.5% of the cases with positive smears yielded positive cultures. The gonococcus could not be cultivated from any case in which gonococci could not be detected on repeated careful smear examination. In cases from which it was impossible to cultivate the gonococcus, when an organism believed to be the gonococcus was present on smears, the bacterial flora was more diverse than in those cases from which positive cultures were obtained; overgrowth of the gonococcus by other bacteria may have been the factor which prevented the isolation of the former in all the cases considered specific.

Immunologic Reactions of Gonococcus.—Seven of the gonococcus strains isolated were used for rabbit immunization. Four of these were children's strains isolated from vaginal cultures. Two of these died before the animals were sufficiently immunized to produce useful serums. The other 2 rabbits produced serums with agglutinin titers of 1:4096 (serum 1) and 1:8192 (serum 2), respectively. Of the 3 strains from adults, 1 (No. 10) was isolated from a gonorrheal tube and the other 2 (Nos. 15 and 16) from urethral cultures; the resulting serums had titers of 1:8192 (serum 10), 1:4096 (serum 15), and 1:1024 (serum 16), respectively. The rabbits were immunized by injections of living washed gonococci. The growth from 1 slant of a 48-hour culture was injected intravenously at 5 day intervals. While there was great variation in the length of time required for immunization, no immunity was manifested until after at least 6 injections.

With the serums prepared as above, the highest titer at which the isolated strains of gonococcus were agglutinated was determined. To varying dilutions of normal rabbit serum and to varying dilutions of each immune serum, an equal amount of living washed bacterial suspension was added. The tests were placed in the hot water bath at 56 C. for 4 hours, and then in an icebox over night, at the end of which time the readings were taken. Whenever there was any clumping in the control tube containing bacterial suspension only, or in any of the tubes containing normal serum and suspension, the tests were discarded. Each immune serum was tested with 6 children's strains (Nos. 1 to 6 inclusive), all from vaginal cultures, and with 5 adults' strains, 2 of which (Nos. 10 and 11) were from gonorrheal tubes, 2 (Nos. 15 and 16) from urethral cultures, and 1 (No. 12) from a vaginal culture. The results of the agglutination experiments are given in table 1, the highest dilution at which agglutination occurred being recorded.

Strain 1 yielded a highly specific monovalent serum. Serum 2 had a wider range of activity than serum 1, but was, in general, more active with the children's strains than with those from adults. Serum 10 appeared to be polyvalent, to a degree comparable with serum 2, and on the whole agglutinated

⁶ Jour. Path. & Bacteriol., 1911, 15, p. 76.

⁷ Jour. Infect. Dis., 1921, 28, p. 133.

⁸ Jour. Path. & Bacteriol., 1922, 25, p. 346.

the strains from children better than those from adults. Serum 15, like serum 1, appeared to be monovalent. Serum 16 also had a narrow range of activity, but agglutinated 1 of the children's strains at a titer equal to that for its homologous strain.

The number of strains which could be submitted to immunologic study was too few to permit any definite conclusions as to grouping. The results obtained do not warrant a separation of strains derived from vulvovaginitis of children from those of adult origin, although they do indicate immunologic differences in

TABLE 1
AGGLUTINATION REACTIONS OF GONOCOCCUS STRAINS

Gonococcal Strains	Serums				
	From Vulvovaginitis Strains		From Adult Strains		
	1	2	10	15	16
From Vulvovaginitis:					
1. Vagina.....	1:4096				
2. Vagina.....	1:8	1:8192	1:1024	1:512	1:1024
3. Vagina.....	1:512	1:32	1:4	
4. Vagina.....	0	1:4	1:8192	1:8	1:64
5. Vagina.....	1:16	1:1024	0	0
6. Vagina.....	1:64	0	1:512	1:32	0
From Adults:					
10. Tube.....	1:8	1:256	1:8192	1:4	1:16
11. Tube.....	1:4	0	1:16	1:4	1:4
12. Vagina.....	0	0	1:256	1:64	1:4
15. Urethra.....	0	1:32	1:16	1:4096	1:4
16. Urethra.....	1:1024

individual strains of diverse origin. Louise Pearce,⁹ who studied the serologic relations between gonococci isolated from male urethritis and those of vulvovaginitis in small girls, concluded that there might be 2 main types of gonococci, although the distinction was only relative.

GRAM-POSITIVE COCCI

Gram-positive cocci appearing on the original plate cultures were transferred to blood agar and then tested for bile solubility. If bile soluble, agglutination tests with type antipneumococcus serums were set up and the type of pneumococcus determined. The pneumococcus was isolated only once; this strain belonged to type 4.

Staphylococci were isolated from 45 cultures. All of these with 2 exceptions, which were *Staphylococcus aureus*, were of the albus variety.

Streptococci: Bile insoluble cocci growing in pairs or chains were again transferred to blood-agar plates and the characteristic of hemolysis or non-hemolysis determined. Transplants were also made to lactose, mannitol and salicin broths and the strains were classified according to Holman's¹⁰ classification; the cultures were incubated for 7 days and the readings recorded at that time. Thirty-seven strains of streptococcus were isolated and studied. Only 4 of the 37 were hemolytic; these fermented lactose, mannitol and salicin (*St. infrequens*). Twenty of the nonhemolytic strains fermented lactose,

⁹ Jour. Exper. Med., 1915, 21, p. 289.

¹⁰ Jour. Med. Research, 1916, 24, p. 377.

mannitol and salicin (*St. fecalis*). Six nonhemolytic strains fermented lactose but not mannitol and salicin (*St. salivarius*). Five fermented lactose and salicin but no mannitol (*St. mitis*). Two did not ferment any of the 3 carbohydrates (*St. ignavus*).

TABLE 2
CLASSIFICATION OF STREPTOCOCCI ISOLATED

Species	No. of Strains	Lactose	Mannitol	Salicin
Hemolytic: <i>St. infrequens</i>	4	+	+	+
Nonhemolytic: <i>St. fecalis</i>	20	+	+	+
<i>St. salivarius</i>	6	+	0	0
<i>St. mitis</i>	5	+	0	+
<i>St. ignavus</i>	2	0	0	0

GRAM-NEGATIVE COCCI

The isolated gram-negative cocci, other than gonococci, did not resemble the latter, in that the former were small and rounder than gonococci and some had a staphylococcus grouping rather than a diplococcus arrangement. Cultural and serologic reactions excluded these from the gonococcus group. As far as they could be identified, 2 strains fell into Hiss and Zinsser's¹¹ chromogenic group 1, which ferments dextrose, maltose, levulose and saccharose, and 2 into the *M. catarrhalis* group.

GRAM-NEGATIVE BACILLI

Gram-negative bacilli which appeared on the original plate cultures were transplanted to Endo's agar plates. Those whose identification was completed fell so regularly into the *B. coli* group, that, as the work progressed, their identification was not carried further than the determination of their gram-staining properties.

GRAM-POSITIVE BACILLI

Gram-positive bacilli occurred so frequently that this group was subjected to further study. Forty-one strains were isolated. Morphologically, they somewhat resembled *B. diphtheriae* but, in general, were shorter and thicker and stained uniformly. In no instance did we find typical polar granules or transverse bands. On blood agar the colonies were small, round and elevated, and grayish white. From their general characters, we have grouped these small gram-positive bacilli together as "diphtheroids."

Hiss and Zinsser¹¹ differentiate *B. diphtheriae* from the somewhat similar *B. xerosis* and *B. hoffmanni* by the use of 2 fermentable substances, saccharose and dextrin. *B. diphtheriae* forms acid from dextrin and not from saccharose; *B. xerosis* forms acid from saccharose and not from dextrin; *B. hoffmanni* does not form acid from either. Our strains, when classified according to their reactions in saccharose and dextrin, could be subdivided as follows: with fermentative reactions like those of *B. diphtheriae*, 10 strains; like *B. xerosis*, 6 strains; like *B. hoffmanni*, 11 strains. In addition, there were 14 strains which fermented both saccharose and dextrin.

From their morphology it was evident that none of the gram-positive bacilli could be true diphtheria bacilli, although 10 strains were like the latter in gram-staining and fermentation reactions. While the use of saccharose and dextrin may serve to differentiate *B. diphtheriae* from the 2 other closely related

¹¹ Textbook of Bacteriology, 1914.

organisms included in Hiss and Zinsser's table, these 2 carbohydrates alone did not permit a fine subdivision of our strains. For further differentiation, the reactions on dextrose and lactose were also studied. By the use of the 4 carbohydrates, 5 main provisional groups could be made: I, fermenting 4 carbohydrates, 11 strains; II, fermenting 3, 9 strains; III, fermenting 2, 10 strains; IV, fermenting 1, 9 strains; fermenting none, 2 strains. Groups II, III, and IV could be still further subdivided, according to the particular carbohydrates fermented (table 3).

TABLE 3
FERMENTATION REACTIONS OF GRAM-POSITIVE DIPHTHEROID BACILLI

	No. of Strains	Dextrose	Saccharose	Lactose	Dextrin
I. All fermented.....	11	+	+	+	+
II. 3 fermented.....	5	+	+	+	0
	2	+	+	0	+
	2	+	0	+	+
III. 2 fermented.....	1	+	+	0	0
	1	+	0	+	0
	7	+	0	0	+
	1	0	+	0	+
IV. 1 fermented.....	8	+	0	0	0
	1	0	0	0	+
V. None fermented.....	2	0	0	0	0

The use of still other fermentable substances would undoubtedly permit still further separation, but would not help to establish inter-relationships to any more satisfactory degree than do the 4 substances used. The group of gram-positive bacilli isolated is probably a heterogeneous mixture of species, which need not bear any relation to each other, except common morphology and staining reactions.

DISCUSSION

The results of our bacteriologic investigation of cases of vulvovaginitis in young girls reporting to the dispensary for treatment divide these cases into 2 main groups: those in which the gonococcus was present and was apparently the important etiologic factor in the condition; and those in which the gonococcus could not be detected or isolated. It is necessary, therefore, to distinguish between a specific gonorrheal infection and a nonspecific process.

In our group of gonorrheal cases, which constituted 35.7% of the total studied, the gonococcus was isolated in culture in 53.3%. In the remainder of the gonorrheal cases, an organism considered to be the gonococcus on the basis of intracellular situation, morphology and staining reaction, was detected in direct smear examinations but could not be cultivated. In this latter group, the bacterial flora, in the direct smears and in the cultures, was much more variegated than in those cases

from which the gonococcus could be cultivated. It was, we have no doubt, the association with large numbers of other bacteria of various species which made isolation of the gonococcus difficult or impossible in such cases. It is possible that the associated mixed flora may be a factor in the inflammatory process, and that cases in which other bacteria predominate over the gonococcus may run a somewhat different clinical course than those in which the gonococcus predominates. Although the newer cultural methods have simplified the isolation of the gonococcus, our failure to isolate the organism in a certain percentage of cases in which the organism was believed to be present in direct smears, leads us to conclude that the smear method as a diagnostic procedure is preferable to the cultural method in office and dispensary work. The gonococcus was not cultivated from any case in which it was not detected in smears. The failure of the cultural method to establish a specific diagnosis in cases negative on repeated direct examination gives to the cultural method no advantage which compensates for its greater technical difficulty.

In the cultural study, no gram-negative coccus which was isolated was considered a gonococcus unless its fermentation reactions were typical and unless it was agglutinated by a polyvalent antigonococcus serum. The serum dilutions in which the recently isolated gonococci were agglutinated by the polyvalent serum varied from 1:8 to 1:8,192. Objection may be made that agglutination in a dilution of 1:8 is not adequate for species diagnosis. But the absence of agglutination in the controls, and the typical fermentation reactions, together with agglutination by the specific serum even in a dilution of 1:8, were considered sufficient to establish the diagnosis.

Comparative agglutination experiments, by means of monovalent antisera, on strains isolated from children and on those isolated from adult women, did not indicate any distinct differences between the 2 groups of strains. The number of strains thus investigated was, however, too few to permit definite conclusions on this point. The relative infrequency of complications in the gonorrheal infections of children as compared with those of adult women is a matter difficult of explanation. If a clear-cut distinction could be made by immunologic reactions between strains from the 2 groups of cases, such a biologic difference might receive weight in explaining the clinical differences. In the absence of such a distinction, local resistance of the nonfunctioning upper genital tract of the female child may be a factor, but one

for which we can offer no absolute proof. The agglutination experiments with the monovalent serums indicated wide differences between individual strains, irrespective of their source. Some of the monovalent serums were highly specific and caused no or only slight agglutination of heterologous strains; other serums had a wider range of activity. A number of fairly distinct strains were represented among those studied, but the strain differences bore no relation to the juvenile or adult origin of the cultures.

As much interest attaches to the nongonorrheal cases, which formed 64.3% of the total, as to those of specific origin. From the sociologic as well as from the etiologic standpoint, the 2 groups should be differentiated. Perhaps the same factors are active in the nonspecific forms of vulvovaginitis as in the gonorrheal variety, so that the same attempts at control by isolation may be necessary. The bacteriologic evidence, however, appears to implicate auto-infection as the chief factor in the nonspecific cases, so that quarantine and exclusion from school would seem to be unnecessary in this group of cases. The same therapeutic measures applied to both groups indicate that the nonspecific cases yield more readily to treatment than do those due to the gonococcus. It is possible that some of the cases which we have placed in the nonspecific group may have been chronic specific cases, in which the gonococcus could no longer be found on smear or cultural examination. But in view of the amount of labor expended in the attempt to detect or isolate the gonococcus in these cases, we are forced to conclude that a nonspecific form of vulvovaginitis occurs and to place in this group all cases in which careful work failed to reveal the gonococcus.

While the bacterial flora may be quite varied in the nonspecific cases, the probable origin of the bacteria found is a matter of considerable interest and gives a clue to the source of the infection. The organisms most frequently encountered were gram-positive cocci, which on culture proved to be staphylococci and streptococci. Lack of cleanliness has always been emphasized as a factor in vulvovaginitis, and the staphylococci isolated, being with only 2 exceptions of the albus variety, may have had a causal relation in some cases, infection occurring by staphylococci from the general body surface. Of the streptococci isolated, the great predominance of strains of intestinal origin is striking. We believe that this is evidence of auto-infection by fecal contamination. The pneumococcus was found only once; this was a type 4 strain.

The small number of gram-negative cocci other than gonococci proved to be either *M. catarrhalis* or chromogenic cocci. In view of the involvement of mucous membranes by the former, it may be a factor in an occasional case. Like *Staphylococcus albus*, it may play a rôle only in those children in whom lack of cleanliness may permit any species of bacterial agent to establish itself.

Gram-negative bacilli of the colon group are to be expected in children of the social stratum from which dispensary cases of vulvovaginitis are derived. Contamination of the vulva and lower vagina by intestinal contents is probably the rule rather than the exception. That the colon bacillus is a frequent cause of acute inflammatory processes in this region is doubtful. When an inflammation is initiated by the irritant action of urinary or fecal matter or by definitely pathogenic bacteria, the colon bacillus may be a factor in maintaining the process.

Gram-positive bacilli of a pseudodiphtheroid type ranked next in frequency to gram-positive cocci and gonococci. From their fermentative reactions they do not appear to form a homogeneous group. Until pathogenic activity is proved for some one or more species of this group, too much importance should not be attached to the presence of gram-positive bacilli. Possibly most of the members of this group have the same significance as the colon bacillus. Further investigation is required to determine the origin of these bacilli, their relation to the intestinal flora, and the pathogenicity of individual species.

In the nonspecific cases of vulvovaginitis, streptococci, especially those of intestinal origin, appear to be the most important offenders. In this entire group, lack of cleanliness and the irritation caused by filth and excretions must be important factors; in fact, in a few of the cases they appeared to be the only ones. Such cases yielded readily to ordinary hygienic measures and required no other local treatment. Neglect of a condition, primarily not infectious in origin, permits the establishment of a bacterial flora which appears to have an active part in maintaining the process and making it worse. Such cases required active local treatment, but as a rule yielded somewhat more readily to the measures applied than did the gonorrheal cases.

SUMMARY

In a series of 42 cases of vulvovaginitis among children reporting to a dispensary clinic for treatment, 35.7% were due to the gonococcus and 64.3% were nonspecific.

In 53.3% of the specific cases, the gonococcus was isolated in pure culture. In the remainder, the specific diagnosis was based on the presence within leukocytes of morphologically typical, gram-negative diplococci.

Although the newer cultural methods have simplified the isolation of the gonococcus, the direct smear examination should be given preference over the cultural method as a diagnostic procedure.

In no case was it possible to cultivate the gonococcus when gonococcus-like bacteria were not detected in the smear examination.

It was impossible, by immunologic methods, to differentiate the gonococci isolated from children from those derived from women.

In the nongonorrheal cases a mixed flora was present, but streptococci of intestinal origin appeared to be the most important agents. Staphylococci, colon bacilli and gram-positive bacilli were frequently encountered, probably as secondary invaders or harmless symbionts.

Uncleanliness and local irritation are believed to be an important, and probably the primary, factor in the nonspecific cases, the condition thus established being maintained or made worse by bacterial localization. In the latter part of the process, bacteria of intestinal origin appear to be most important.

Cases in which filth and irritation appeared to be the chief factors yielded most readily to the routine therapeutic procedures which were applied in all cases. The gonorrheal cases were most stubborn. In an intermediate position, as regards the readiness with which they yielded to treatment, were the cases in which local irritation was followed by the localization of bacteria other than the gonococcus.

Quarantine and exclusion from school are not believed to be necessary in the nongonorrheal cases.

BIOLOGIC AND SEROLOGIC STUDIES OF BACILLUS MUCOSUS GROUP

COMPARISON OF STRAINS FROM GRANULOMA INGUINALE WITH STRAINS FROM RESPIRATORY TRACT

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In the course of cultural studies of the lesions of inguinal granuloma,¹ encapsulated gram-negative bacilli have been encountered. Preliminary cultures of strains of encapsulated bacilli from the lesions of inguinal granuloma indicated that they belonged to the mucosus group, and since members of this group have been found frequently in lesions of the respiratory tract, it was considered desirable to make a comparative study of a number of strains of respiratory origin and available strains of granuloma origin. Two stock strains of *B. aerogenes* and one of *B. rhinoscleromatis* were carried through the various procedures as controls for comparison with the freshly isolated strains.

In all, 27 strains were used, including 8 strains of encapsulated bacilli of granuloma origin, 13 strains of respiratory origin, 3 somewhat atypical strains of granuloma origin, 2 stock strains of *B. aerogenes* and 1 stock strain of *B. rhinoscleromatis* (table 1). Four general lines of procedure were followed: (1) a study of morphology, (2) a study of the biochemical reactions, (3) a study of the inhibition of growth by tartar emetic, and (4) a study of the serologic reactions.

CHARACTERS OF GROWTH AND MORPHOLOGY

The surface growth on glucose agar of the different strains was in all instances characteristic of the mucosus group. The colonies grew to a large size after 24 hours' incubation and appeared as gray-white, glistening, dome-shaped elevations on round regular bases from 1 to 3 mm. in diameter. When touched with wire, the colonies appeared viscid. If the plate cultures were incubated in an inverted position, frequently after 48 hours portions of the large viscid colonies would run down on the lid of the dish. The colonies by transmitted light were of a slightly brownish tinge, and by transmitted light before an electric light, or in the direct sunlight, the thinner portions of the colonies exhibited a brilliant iridescence. In the older colonies there was the

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¹ Randall, Small and Belk: Jour. Urol., 1921, 5, p. 539; Surg., Gynec. & Obst., 1922, 34, p. 717.

tendency toward the formation of streaked, irregular opacities producing an effect similar to streaks of purulent material in nasal mucus.

After continuous cultivation over periods of months, strains F1, F2, F3, F5, F8, F10, F13, F15, G7, and R1 retained these characters when grown on moist glucose agar. Strains F6, F11, F12, A1, A2, G1, G2, G3, G4, G5, G6, and G8, under the same conditions, failed to retain the extreme mucoid properties exhibited when isolated from the lesions. The colonies on glucose agar came to appear more opaque, white and flat, but retained moist and glistening surfaces.

Strains G9, E.P.v., and M3B1, from the first, grew as regularly rounded, dome shaped, moist, white colonies, but they had a firmer consistency than that of the markedly mucoid strains. By transmitted light from a strong source, there appeared a marked iridescence throughout the smaller colonies and about the edges of the larger ones. The latter feature was by far the most striking character of this type of organism.

TABLE 1
ORIGIN OF STRAINS

Strains	Origin
F1, F2.....	Tonsil
F3, F5, F8, F11, G7.....	Heart blood, pneumonia
F6, F9, F13, F15, F16.....	Sputum, pneumonia
F10.....	Lung, pneumonia
F12.....	Feces, pellagra
G1, G2, G3, G4, G6, G8, G9, M3B1, E.P.V.....	Inguinal granuloma
G5.....	Anal papilloma
R1.....	<i>B. rhinoscleromatis</i> , stock culture
A1, A2.....	<i>A. aerogenes</i> , stock culture

All were strains of gram-negative bacilli. With capsule stains there was little difficulty in demonstrating capsules, which varied in width depending on the gross character of the growth. The bacilli of the first two classes mentioned were short and thick, but in the older cultures, especially in glucose, they showed a tendency to appear in long irregular forms. In the strains of the last type mentioned, the organisms in the young cultures appeared as short, thick, coccoid-like bacilli, at times with difficulty recognizable as bacilli rather than cocci. In the older cultures, however, they were definitely recognizable as short thick bacilli with rounded ends, and tending to be more uniform in size and shape than those of the first two types considered.

With Wright's stain, the capsules were easily demonstrated in all instances, provided the differentiation with water was not excessive, in which case the capsules would be entirely destained. With the proper degree of differentiation, the capsules appeared either a pale blue or a clear pink, while the body of the bacillus stained a dark blue. The bacilli in young cultures on glucose agar with Wright's stain appeared identical with the organisms within the cytoplasm of the large mononuclear cells of the exudate of the lesions of granuloma inguinale. With the strains of the third type, this resemblance was most marked since these organisms did not tend to grow long forms in cultures. If, after staining with Wright's stain, the preparations were differentiated in water to the extent that the capsules were entirely destained, the bacilli showed polar, usually bipolar, collections of chromatin, stained a deep blue, while the intervening body shaft stained a pale blue. A few of the bacilli always stained a deep blue throughout.

BIOCHEMICAL REACTIONS

The biochemical tests used were of two general types: (1) a study of carbohydrate fermentation reactions, and (2) a study of the manner of the utilization of nitrogenous compounds. Some of the tests have been developed within recent years and consequently were not used by former investigators in their studies of this group of bacteria. Other tests, employed by them as well, have furnished data useful for orientation and interesting for comparative analysis.

In studying the fermentation reactions, 18 carbohydrates were used. Cultures were grown in a beef extract broth containing 1% peptone, and 1% of the carbohydrate designated. The medium was inoculated from young agar cultures and incubated for a period of 5 days at 37 C. At the end of this period, gas production was recorded, and the changes in the hydrogen-ion concentration were determined by the method of Clark and Lubs (table 2).

An analysis of the results in table 2, shows that three sugars, dextrose, lactose, and sucrose, offer the best possibilities for a classification. According to the reactions with these sugars the strains may be arranged in groups as follows:

1. Strains which ferment dextrose, lactose and sucrose,
 With formation of gas: G1, G2, G8, F3, F6, F8, F10, and A1.
 With or without formation of gas: G6, F9, F13, F15 and A2.
2. Strains which ferment dextrose and lactose (but not sucrose);
 With formation of gas: G3, G4, and G5.
 With or without formation of gas: G7, F2, F5, F12, and R1.
3. Strains which ferment dextrose (but not lactose nor sucrose),
 With formation of gas: F1 and G9.
 With or without formation of gas: F11 and F16.
4. Strains which ferment neither dextrose, lactose nor sucrose: E.P.v. and M3B1.

These groups are constituted by granuloma and respiratory strains alike, so that as far as the carbohydrate reactions serve to indicate, there appears to be no differentiation between the two. Strains G6 and F11 failed to produce gas in any of the carbohydrates fermented, and in this respect they appear to resemble *B. rhinoscleromatis*. These strains were obtained from lesions unrelated to rhinoscleroma, so that inability to produce gas apparently cannot safely be considered a differential character of *B. rhinoscleromatis*. The *B. aerogenes* strain A2 failed to ferment dulcitol, a variation raising a question as to its real identity.

These results do not entirely conform with the results of previous investigators. Using dextrose, lactose and sucrose, Clairemont² found that when fermentation occurred both acid and gas were produced in all 3 of these sugars alike, except in the cases of 2 strains which produced gas in dextrose and lactose but which fermented sucrose without gas formation. Perkins³ divided the encapsulated bacilli of the mucosus type into 3 groups according to the reactions on dextrose, lactose and sucrose. Group 1 was represented by organisms fermenting all 3 sugars with acid and gas formation; group 2 by organisms fermenting dextrose and sucrose with acid and gas; and group 3 by organisms fermenting dextrose and lactose with acid and gas formation.

² Ztschr. f. Hyg. u. Infektionskr., 1902, 39, p. 1.

³ Jour. Infect. Dis., 1904, 1, p. 241.

TABLE 2

FERMENTATION OF CARBOHYDRATES

Carbohydrate	G1	G2	G3	G4	G5	G6	G7	G8	F1	F2	F3	F5	F6	F8	F9	F10	F11	F12	F13	F15	F16	EPv	M3B1	G9	A1	A2	R1	Control	
Dextrose.....	+ 4.5	+ 4.4	+ 4.4	+ 4.4	+ 4.3	- 4.8	- 4.8	+ 5.2	+ 4.4	+ 4.4	+ 4.2	+ 4.4	+ 4.6	+ 4.3	+ 4.3	+ 4.4	6.3	- 4.6	- 4.6	+ 5.0	- 4.6	- 8.0	-	- 7.9	+ 4.9	+ 4.5	- 4.4	- 4.6	- 7.4
Levulose.....	+ 4.4	+ 4.7	+ 4.7	+ 4.4	+ 4.5	- 4.3	+ 4.3	+ 4.7	+ 4.5	+ 4.7	+ 4.6	+ 4.6	+ 4.6	+ 4.6	+ 4.6	+ 4.3	6.9	+ 4.5	+ 4.4	+ 4.5	- 4.6	- 6.9	- 7.0	- 6.9	+ 4.7	+ 4.7	+ 5.0	- 7.5	
Galactose.....	+ 4.9	+ 4.6	+ 4.6	+ 4.3	+ 4.0	- 4.8	- 4.8	+ 5.2	+ 4.7	+ 4.7	+ 5.2	+ 5.0	+ 4.5	+ 4.6	+ 5.1	+ 4.4	4.4	+ 4.0	+ 4.5	+ 5.0	- 5.0	- 7.6	- 7.6	- 8.0	+ 4.7	+ 4.8	+ 5.3	- 7.5	
Lactose.....	+ 4.4	+ 4.3	+ 4.7	+ 4.9	+ 5.0	- 5.1	- 5.1	+ 4.8	- 7.5	- 6.4	+ 5.2	- 5.3	+ 4.5	+ 5.2	- 5.3	+ 4.9	7.2	+ 4.8	- 5.2	- 5.6	- 7.6	- 8.0	- 8.0	- 8.0	+ 4.4	+ 5.6	+ 6.2	- 7.5	
Sucrose.....	+ 4.5	+ 4.4	- 8.0	- 8.0	- 5.0	- 7.6	- 7.6	+ 5.2	- 8.0	- 8.0	+ 4.4	- 8.0	+ 4.7	+ 4.4	+ 4.6	+ 4.4	8.0	- 8.0	+ 5.0	+ 5.1	- 7.6	- 8.0	- 8.0	- 8.0	+ 4.5	+ 4.5	+ 7.3	- 7.5	
Maltose.....	+ 4.9	+ 4.7	+ 4.7	+ 4.2	+ 4.3	- 4.5	- 4.5	+ 5.2	+ 4.6	+ 4.5	+ 5.1	+ 4.6	+ 4.7	+ 4.7	+ 4.6	+ 4.2	7.3	+ 4.2	+ 4.2	+ 4.2	+ 4.2	- 7.7	- 7.7	- 7.7	+ 4.6	+ 4.7	+ 4.9	- 7.5	
Rhamnose.....	+ 4.8	+ 4.6	+ 5.0	+ 4.4	+ 4.6	- 5.1	- 5.1	+ 4.4	+ 4.8	+ 4.8	+ 4.9	+ 4.7	+ 4.6	+ 4.7	+ 4.4	+ 4.4	7.0	+ 4.5	+ 4.7	+ 4.9	- 7.2	- 7.2	- 7.6	- 7.6	+ 5.0	+ 5.6	+ 5.1	- 6.8	
Arabinose.....	+ 4.4	+ 4.5	+ 4.5	+ 4.1	+ 4.0	- 4.2	- 4.2	+ 4.1	+ 4.5	- 4.1	+ 4.6	+ 4.5	+ 4.3	+ 4.3	+ 4.4	+ 4.0	4.0	+ 4.0	+ 4.1	+ 4.4	- 4.0	- 6.6	- 6.4	- 6.6	+ 4.5	+ 4.7	+ 4.7	- 6.8	
Raffinose.....	+ 5.7	+ 5.4	- 7.0	- 7.5	- 7.8	- 5.3	- 5.3	+ 6.4	- 5.1	+ 5.3	+ 5.3	+ 5.3	+ 5.3	+ 5.2	+ 5.2	+ 4.8	7.5	- 7.8	- 4.9	+ 5.5	- 6.9	- 7.2	- 7.9	- 7.9	+ 5.0	+ 6.0	+ 5.6	- 6.8	
Xylose.....	+ 4.4	+ 5.4	+ 4.2	+ 4.1	- 7.2	- 4.2	+ 4.2	+ 4.2	+ 4.2	+ 4.4	+ 4.8	+ 4.8	+ 4.2	+ 4.4	+ 4.7	+ 4.2	4.2	+ 4.1	+ 4.3	+ 4.6	- 4.2	- 7.1	- 7.1	- 7.1	- 7.1	- 4.4	- 5.6	- 4.8	- 6.8
Adonitol.....	+ 4.5	+ 4.4	- 8.0	- 7.4	- 7.8	- 4.5	- 4.5	+ 6.2	+ 4.2	+ 4.3	+ 4.5	+ 4.6	+ 4.5	+ 4.4	+ 4.5	+ 6.2	7.5	- 7.5	+ 4.2	+ 4.2	- 7.4	- 7.7	- 7.7	- 8.0	+ 4.8	+ 4.4	+ 5.7	- 7.4	
Mannitol.....	+ 4.3	+ 4.5	+ 4.4	+ 4.8	+ 4.9	- 7.3	- 7.3	+ 6.6	+ 4.4	+ 4.4	+ 4.5	+ 4.4	+ 4.4	+ 4.4	+ 4.3	+ 4.9	8.0	+ 4.6	+ 4.9	+ 4.6	- 7.9	- 7.8	- 8.0	- 7.8	- 7.8	+ 4.4	+ 4.9	- 7.4	
Dulcitol.....	- 8.0	- 8.0	- 8.0	- 7.9	- 5.2	- 8.0	- 7.9	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	- 4.9	7.9	- 7.9	- 7.9	- 7.9	- 7.9	- 7.8	- 7.8	- 7.8	- 7.9	- 8.0	- 7.9	- 7.5	- 7.5
Glycerol.....	+ 4.4	+ 4.7	- 7.2	- 5.4	- 5.8	- 4.8	- 4.8	+ 4.8	+ 4.6	+ 4.5	- 7.3	- 7.3	+ 4.5	- 7.0	- 7.3	+ 4.6	7.8	- 5.3	- 5.0	- 6.4	- 8.0	- 8.0	- 7.9	- 7.9	+ 4.6	+ 6.9	+ 6.2	- 7.5	
Inulin.....	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	- 6.6	- 8.0	- 8.0	- 8.0	- 4.5	- 7.9	- 8.0	- 8.0	- 8.0	- 8.0	- 8.0	8.0	- 8.0	- 7.5	- 8.0	- 8.0	- 7.8	- 8.0	- 8.0	- 8.0	- 7.6	- 7.9	- 7.5	- 7.5
Sorbitol.....	+ 4.3	+ 4.4	- 8.0	- 8.0	- 8.0	- 7.6	- 7.6	+ 5.2	+ 4.2	+ 4.4	+ 4.3	+ 4.6	+ 4.5	+ 4.3	+ 4.4	+ 4.4	8.0	- 8.0	+ 5.0	+ 5.1	- 4.6	- 8.0	- 8.0	- 8.0	+ 4.4	+ 4.4	+ 4.7	- 7.5	
Dextrin.....	+ 4.4	- 8.0	- 7.7	- 5.0	- 6.4	- 4.2	- 5.2	+ 4.2	- 8.0	- 7.2	- 7.3	- 8.0	- 7.5	- 7.5	- 7.5	+ 4.2	7.6	- 6.4	+ 4.0	+ 4.8	- 7.3	- 7.7	- 7.8	- 7.7	- 8.0	- 7.7	- 4.8	- 7.5	
Starch.....	+ 4.4	- 5.1	- 8.0	- 6.8	- 7.4	- 5.0	- 6.4	- 6.6	- 8.0	- 8.0	- 5.0	- 8.0	- 4.7	- 8.0	- 6.2	+ 4.2	7.6	- 6.4	+ 4.0	+ 4.8	- 7.3	- 8.0	- 7.8	- 7.8	- 8.0	- 8.0	- 8.0	- 8.0	- 7.6

Gas formation indicated by plus (+) sign; minus (-) sign = no gas formation; the figures represent the hydrogen-ion readings.

More recently, Fitzgerald,⁴ has presented evidence to show that the fermentation of carbohydrates is not dependable as a basis for classification within this group of bacteria. His results indicated that the fermentation reactions of the mucosus group were highly variable and unreliable.

Although the grouping as suggested by the carbohydrate reactions in this investigation depends on the same 3 sugars which were used by both Clairemont and Perkins, it differs from that offered by each of these workers. It differs from Clairemont's study in that acid and gas were not produced in all 3 sugars when fermentation occurred. It differs from Perkins' study in that gas production alone apparently cannot include all of the mucosus strains. A fourth group is demonstrated here, the strains of which ferment neither dextrose, lactose nor sucrose. In general, the great variability of the fermentation reactions, even when only 3 sugars are considered, tends to support the contention of Fitzgerald that this means of study is not dependable as a basis for classification of organisms of this general type. From the 18 carbohydrates used, dextrose, lactose and sucrose are the only ones yielding differential reactions within the group, and the adaptation of the reactions with these 3 within the small group of strains considered serves to offer more confusion than it does system. The evidence presented serves best in demonstrating the fallacies of trying to employ carbohydrate fermentation reactions in the identification and classification of bacteria of this group, and emphasizes the importance of a search for more reliable methods.

UTILIZATION OF NITROGENOUS COMPOUNDS

The manner of the utilization of nitrogenous compounds by members of the mucosus group was investigated by means of the following tests: formation of ammonia; reduction of nitrates to nitrites; production of indol and creatinin from peptone; reactions on gelatin and litmus milk; methyl red and Vosges-Proskauer reactions; and the amino-acid decomposition of peptone.

Tests for ammonia production were made on cultures grown for a period of 1 week at 37 C. in a medium containing 1% peptone, 0.5% sodium chloride, and 0.5% dibasic potassium phosphate. The Nessler reagent was used for these tests.

For the nitrate reduction reaction, a medium composed of 1% peptone, 1% potassium nitrate, and 0.5% dibasic potassium phosphate, was used. The cultures and the controls were incubated at 37 C. for a period of 1 week, when tests for nitrite were made by the sulphanilic acid-alpha-naphthylamine method.

For the production of indol, a 1% peptone-0.5% sodium chloride medium was used. The cultures were incubated at 37 C. for a period of 10 days, test specimens being removed on the 5th, the 7th, and the 10th days, and tested for the presence of indol by the para-dimethyl-benzaldehyde method.

Tests for creatinin were made by the picric acid method on the cultures after a period of 3 weeks' incubation at 37 C.

Vosges-Proskauer and methyl red tests were made in the same manner as in the sanitary analysis of water.

Amino-acid determinations were made on cultures in a medium of 1% peptone (Difco) after 1, 3, 5, 9, 12, and 15 days' incubation at 37 C. The formol titration method of Sorensen was used.

The results of these experiments are given in table 3.

⁴ Ibid., 1914, 15, p. 268.

TABLE 3
THE UTILIZATION OF PROTEIN

	G1	G2	G3	G4	G5	G6	G7	G8	F1	F2	F3	F5	F6	F8	F9	F10	F11	F12	F13	F15	F16	FPv	M3B1	G9	A1	A2	R1	Control
Motility.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—
NH ₃ formation.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	+	+	+	—
(NO ₃) reduction.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	+	+	+	—
Indol.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Creatinin.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Milk.....	D	D	AC	AC	AC	AC	AC	AC	A	—	±	AC	AC	—	—	AC	AC	AC	A	A	A	—	—	—	AC	±	—	—
Gelatin.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Methyl red.....	+	—	—	+	+	+	+	—	+	+	±	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Voges-Proskauer.....	—	—	+	—	—	—	—	+	—	+	+	+	—	—	+	—	—	—	—	—	+	—	—	—	+	+	—	—
Amino-acid:																												
1st day.....	24	32	36	24	32	32	32	32	28	36	36	48	40	32	24	36	40	40	28	20	28	22	32	40	28	40	40	26
3d day.....	32	64	40	36	32	40	32	40	52	36	36	22	14	36	36	32	36	28	36	28	28	20	32	28	36	40	26	26
6th day.....	44	72	78	40	40	44	52	52	40	60	52	80	48	48	40	48	40	48	40	41	41	36	40	40	44	52	48	26
9th day.....	32	56	44	44	44	40	41	40	41	64	52	56	48	14	48	44	40	44	36	32	40	48	36	44	40	60	44	26
12th day.....	36	52	44	40	44	40	48	40	40	48	—	41	—	40	—	40	40	40	40	40	41	36	44	40	44	48	40	26
15th day.....	40	72	40	40	32	44	32	32	44	—	41	40	40	40	—	36	40	40	48	48	44	40	44	—	44	40	40	26

Under milk, D indicates digestion; C, coagulation; and A, acid.
Under amino-acids, the figures represent the number of c. c. of N/20 NaOH required to neutralize 100 c. c. of the culture.

A study of table 3 shows:

1. Each of the strains formed ammonia. In the cases of G8, G9, E.P.v. and M3B1 the amounts of ammonia formed were considerably less than those formed in the other cultures.

2. Nitrates were reduced in every instance, but here again strains G9, E.P.v. and M3B1 were much less active than were the other strains.

3. Tests for the presence of indol were uniformly negative.

4. In none of the cultures was creatinin produced.

5. In litmus milk after 1 week, 3 strains produced acid, coagulation and beginning peptonization; 16 strains produced acid, usually sufficient for coagulation; 4 strains showed only a slight acidity; and 4 strains showed no visible change in the reaction. Strains G9, E.P.v. and M3B1 again gave evidence of feeble activities. Acid production closely paralleled the acid production in lactose broth.

6. None of the strains liquefied gelatin after a 14-day incubation period at 20 C.

7. The Vosges-Proskauer and the methyl red reactions were of no value in this study. In general, there was a greater tendency toward the production of a positive methyl red test than toward the production of a positive Vosges-Proskauer test, but no strict correlation between the 2 tests was evidenced. Strains G2, G9, R1, E.P.v. and M3B1 were negative for both tests, while the 2 aerogenes strains ran true to form with negative methyl red and positive Vosges-Proskauer reactions. E.P.v. and M3B1 were shown not to ferment dextrose, and consequently would be expected to give negative methyl red tests.

8. In the production of amino-acids, the granuloma, respiratory and the rhinoscleroma strains reached the maximum concentration on the 6th day, and the aerogenes and the atypical granuloma strains on the 9th day.

No differentiation between the granuloma and the respiratory strains can be made by any of the tests which were used. In the case of the amino acid decomposition, maximum production appears on about the 6th day for the respiratory, granuloma and the rhinoscleroma strains; and on about the 9th day for the aerogenes and the atypical granuloma strains. Little significance can be attached to this slight difference in the rates of amino acid production.

As compared with the results recorded in the literature on this subject, the ammonia, nitrate, creatinin, Vosges-Proskauer, and the methyl red experiments corroborate the work of Fitzgerald.⁴ Regarding indol formation, Clairemont² found that 3 of 36 cultures formed indol. Perkins³ stated that all but 3 of his cultures produced indol, while Fitzgerald assigns indol formation to only a few of his strains. The results in litmus milk do not differ materially from results reported by these workers. In the case of gelatin liquefaction, Fitzgerald alone found some strains capable of liquefying gelatin. The question of the possible impurity of such cultures must be considered in the light of all the other work to the contrary.

INHIBITION OF GROWTH IN CULTURE BY TARTAR EMETIC

Observations of the inhibitory effect of tartar emetic on the growth of these micro-organisms in cultures were suggested because tartar emetic (as well as some other compounds of antimony) acts as a specific in the treatment of the lesions of inguinal granuloma. This information was desired because of

the bearing that it might have in a consideration of the etiologic relationship of these strains of granuloma origin to inguinal granuloma.

The tests were made in a medium of 1% peptone in water, to portions of which the varying amounts of tartar emetic were added. The tubes were inoculated with a loopful of a 24-hour broth culture of the organism to be studied and incubated at 37 C. for a period of 48 hours. Readings were made of the turbidity from bacterial growth. In the higher concentrations of tartar emetic, a turbidity arising from partial precipitation of the chemical appeared. This was recognized by the microscopic examination of stained preparations from the cultures, and was controlled by carrying sets of uninoculated tubes containing the different dilutions of tartar emetic. Inoculated tubes of the medium to which no tartar emetic had been added served as normal controls of growth of the bacteria under the conditions of the experiment. As an aid in the interpretation of the findings, members of some other groups of bacteria were tested in the same manner for inhibition of growth by tartar emetic.

In table 4, the estimations of the turbidity developing from bacterial growth after 48 hours' incubation are recorded, using the sign (—) to indicate no growth, and the numerals 1, 2, 3, 4, to indicate the relative grades of bacterial turbidity developing.

The striking feature of the results is that the granuloma strains appeared more tolerant of tartar emetic than any other of the bacteria tested, full growth occurring in some instances in dilutions of 1:400, and some growth of all except strains G6 and G7 appearing in dilutions of 1:1,000. The strains of respiratory origin were the most sensitive to the effects of tartar emetic, perceptible growth not appearing until dilutions of 1:10,000, or 1:20,000 were attained. Exceptions were strains F10, F11, and F12. The aerogenes and rhinoscleroma strains, and the staphylococcus were affected to much the same extent as the mucosus strains of respiratory origin. *B. coli* was inhibited slightly more than were the granuloma strains, while *B. pyocyaneus* appeared somewhat more sensitive to the effects of tartar emetic than was *B. coli*.

SEROLOGIC REACTIONS

Agglutination.—Difficulties in preparing agglutinating serums for mucosus strains have been recorded as the common experience of other workers.⁵ Failure to obtain strong agglutinating serums might be attributed to an inability on the part of the antigens used to excite the formation of agglutinins in the serum of animals. A large share in this failure, however, appears to be attributable to the character of the bacterial suspensions used in the agglutination reaction. Suspensions from strains which in cultures retain wide bacterial capsules definitely resist agglutination with specific serum. Such suspensions, especially in concentrated forms, are easily shown to be rich in the mucoid capsule substance, which on the basis of its physical nature alone so tends to increase the viscosity of the menstium as to be a large factor in preventing agglutination. The possibility of the capsule substance acting as a protective colloid tending to prevent flocculation of the bacteria must also be considered. The latter effect could as readily be attributed to the dissolved capsule substance as to the intact capsules. A certain amount of the capsule, however, remains intact about the bodies of the bacilli in the antigen suspensions and here must be regarded as exerting a rôle similar to its defense function in the physiologic reactions of the growing bacteria,

⁵ Defalle: Ann. de l'Inst. Pasteur, 1902, 16, p. 595; Coulter: Jour. Exper. Med., 1917, 26, p. 763.

TABLE 4
INHIBITION OF GROWTH BY TARTAR EMEIC

[illegible]

Reading the turbidity developing in the control in each case as "4", in the dilutions of tartar emetic, 0 represents no growth; 1, 2 and 3 the relative grades of inhibition.

Strains "C", "S", and "P" represent cultures of *B. coli*, *Staphylococcus aureus*, and *B. pyocyaneus*, respectively.

TABLE 5
AGGLUTINATIONS

[illegible]

namely, that of isolating the bacillus more or less effectively from the untoward extraneous influences in the medium. This again, a pure physical effect, appears possible because of the chemical nature of the capsule substance. Rettger⁶ studied the chemical nature of the capsule of the Friedländer bacillus, and regarded it as a glycoprotein related to mucin, but since it differed in some respects from true mucin, he considered it a pseudomucin. Kramer⁷ showed that the capsule substance of the pneumobacillus of Friedländer contained galactan, which on inversion yielded galactose. He stated further that this substance is apparently manufactured by the bacterial cell in the absence of any carbohydrate in the medium. We have no evidence that glycoproteins of this nature are antigenic. At any rate, the intact capsules serve to isolate the antigenic protein of the bacillary bodies from the antibodies in the immune serum.

To eliminate the inhibitory effects of the capsule substance in agglutination experiments, several expedients have been devised by different workers. Porges⁸ devised the method of hydrolyzing the capsule substance by heating suspensions of the bacteria in weak concentrations of hydrochloric acid, afterward neutralizing with sodium hydroxide and proceeding with the agglutination. Streit⁹ objected to this procedure because it led to a tendency to spontaneous clumping and to false agglutination in normal serums. Our trials of this method have convinced us that it is unreliable because of the production of these same effects. Beham¹⁰ met with some success in eliminating bacterial capsules by growing cultures at low temperatures (8 to 12 C.), by growing them on potato medium, and by artificial selection of the smaller and drier colonies.

In the preparation of antigens for our work, we have resorted to prolonged cultivation on plain agar as a means of eliminating capsules. The cultures were grown for from 18 to 24 hours at 37 C. and then stored in the icebox (6 to 10 C.) during the intervals between transplants. The transplants were made at monthly intervals, and the strains were carried in this manner for from 12 to 24 months before use in agglutination reactions. This procedure was not effective in eliminating capsules in all instances, but with the majority of the strains it was successful.

Three methods were used for the preparation of antigens. The turbidity of the antigen in each instance was adjusted to that of the Oxford standard typhoid antigen. (1) The bacteria were grown on agar at 37 C. for from 18 to 24 hours, washed off and suspended in salt solution, adjusted to the turbidity standard, preserved with 0.1% formalin, and stored in the icebox. (2) The bacteria were grown in 1% peptone to which 0.5% sodium chloride was added for from 18 to 24 hours, when the suspensions were adjusted to the turbidity standard, 0.1% formalin added, and stored in the icebox. (3) Cultures were prepared in peptone solution in the same manner and the living organisms were used at once in the agglutination tests.

These methods produced antigens differing in minor particulars one from the others. The cultures in the peptone solution appeared to be more readily agglutinable than were the suspensions in salt solution. The cultures in peptone solution when treated with formalin and stored in the icebox, after

⁶ Jour. Med. Research, 1903, 10, p. 101.

⁷ Centralbl. f. Bakteriöl., I, O., 1921, 87, p. 401.

⁸ Wien. klin. Wchnschr., 1905, 18, p. 691.

⁹ Centralbl. f. Bakteriöl., I, O., 1906, 40, p. 709.

¹⁰ Ibid., 1912, 66, p. 110.

several weeks, showed in some instances tendencies toward spontaneous clumping, which rendered them unfit for further use. On the whole, the living cultures in peptone solution proved the most satisfactory.

The serum dilutions were made 1:10, etc., as high as was necessary to cover the agglutination zone of the serum in question. They were made in 0.9% NaCl solution and in the 1.0% peptone-0.5% NaCl solution. Of these two, the latter was found to be the better for facilitating agglutination. Unfortunately, no study of the effects of the hydrogen-ion concentration was made in these agglutination reactions.

The tests were made in the water bath at 50 C. with the depth of the water so adjusted that the level of the liquid in the agglutination tubes stood out somewhat above the surface of the water in the bath, thus bringing into play the slight convection currents within the tubes which appear so effective in the development of sharp end results in the agglutination reaction. Readings were made after 6 hours, and confirmed after 18 hours in the bath.

As indicated, the antigens differed somewhat in their sensitiveness, and the serum titers were slightly increased by using the peptone salt solution for making the serum dilutions. These effects, while increasing the agglutination titers, did not produce discrepancies in the groupings observed with several of the serums. The results in table 5 represent the agglutination titers of the serums with the most effective method, namely, that of using young living cultures in the 1% peptone-0.5% NaCl solution as antigen added to the serum dilutions in the same peptone-salt solution, the results being read after 18 hours in the water bath at 50 C. The figures represent the highest dilutions of the serum at which definite agglutination was observed with the naked eye.

Nine agglutinating serums were prepared and used with the 29 strains. Eight of these 29 strains were not agglutinated by any of the 9 serums; 6 were agglutinated, usually only in high concentrations of the serum, by only one of the 9 serums; while the remaining 15 strains showed a tendency to group agglutination, each by 3 or 4 of the immune serums. Serums F6, F10, G2, G3, G8 and A1 were effective in producing greatest agglutination of the latter strains, but from the irregular cross-agglutination reactions, these cannot be assembled into one immunologic group.

Strains F1, F2, and F13 were agglutinated by F2 serum only, and not in high dilutions. The same serum, on the other hand, agglutinated A1, G11, G12, and G13, which showed a tendency to be agglutinated by a number of the other serums as well. Antigens G11 and G12 were agglutinated in much higher dilutions of serum F2 than was the homologous antigen of this serum. Strains F1, F2 and F13 had retained their mucoid capsules on prolonged cultivation, while strains G11 and G12 had lost this character. It would at first appear that a titer of 1:2,560 for antigen G12 in a serum which agglutinates its own antigen only 1:80 might depend on the fact that the bacilli of strain G12 did not retain their capsules while those of strain F2 did. In a general way, comparison of the agglutination titers with the growth characters of the different strains shows that those strains not retaining capsules agglutinate in the highest titers, and are the most prone to be affected by group agglutinins. While this may be a mere coincidence, nevertheless, it is emphasized because it develops later from the absorption reactions that group agglutinins are observed for the most part only with the capsule-free bacteria.

Absorption of Agglutinins.—The possibility presented itself that while agglutination might be interfered with by the presence of capsules, the ability of

absorbing agglutinins might not be influenced—a condition much the same as that obtaining when bacteria are suspended in a salt-free menstrum, where agglutination does not occur but absorption of agglutinins occurs regularly.

For the absorption, turbid suspensions were prepared by washing 24-hour growths of each of the strains from plain agar surfaces with normal salt solution. The absorption reaction period was 2 hours in the incubator at 50 C., followed by 18 hours in the icebox. The minimal absorbing dose of the antigen for its homologous serum was determined by a titration method. Since only 9 serums were available for the group of 29 strains, a rough preliminary division of these strains into groups represented by the immune serums was attempted by exposing each of the serums to each of the 29 antigens used in doses, as measured by turbidity standards, which corresponded to 5 times the minimal absorbing dose of the homologous antigen. After such exposures for absorption, the serum was tested for effects on its titer for its own antigen. The results are presented in table 6, and were such that we did not deem it fruitful to plan more elaborate absorption tests.

TABLE 6
ABSORPTION OF AGGLUTININS

Serums	Absorbed by Strains											G4, G5, G6, G7, F3, F5, F8, F9, F11, F12, F15, EPV, M3B1, G9, A2, G11, G12, G13
	G1	G2	G3	G8	F1	F2	F6	F10	F13	A1	R1	
F2.....	0	0	0	0	+	+	0	0	+	0	0	0
F6.....	0	0	0	0	0	0	+	0	0	0	0	0
F10.....	0	0	0	0	0	0	0	+	0	0	+	0
G1.....	+	0	0	0	0	0	0	0	0	0	0	0
G2.....	0	+	0	0	0	0	0	0	0	0	0	0
G3.....	0	0	+	0	0	0	0	0	0	0	0	0
G8.....	0	0	0	+	0	0	0	0	0	0	0	0
A1.....	0	0	0	0	0	0	0	0	0	+	0	0

Two groups—one comprising strains F1, F2, and F13; the other, F10 and R1—are revealed by the absorption tests. The remaining 6 serums were prepared from individual strains so far as this series is concerned. It is noteworthy that strains G11 and G12, which were agglutinated in high dilutions by serum F2, were inactive in removing the agglutinins for F2. Strains G11 and G12 did not retain capsules, which might interfere with the absorption of agglutinins; the reason for this discrepancy must be searched for elsewhere. To ascribe it to group agglutinins would be begging an explanation. The clumping reactions for F1, F2 and F13 with serum F2 more closely resembled precipitin reactions than they did agglutination reactions, as the volume of the clumped mass was larger than that to be expected from the turbidity of the antigens. The reactions stopped abruptly in the series of serum dilution tubes, i. e., dilutions 1:80 or 1:160 showed complete clumping into one voluminous mass, while the tube containing the next higher dilution showed absolutely no clumping. There was not that gradual "shading off" of the clumping which is noticed even in serums of low titer which do not contain interfering precipitins. This phenomenon was observed only with the serums of those strains which retained capsules in cultures. Subsequently, in con-

sidering the precipitin reactions, it will be noted that the capsule-bearing strains were the most favorable for this reaction. The possibility then presents itself that the capsule substance may have another action aside from its physical interference with the normal action of agglutinins, namely, that it is concerned in the formation of precipitins, which latter mask the action of agglutinins both in the direct reaction and in absorption of agglutinins. For example, in this instance the grouping F1, F2 and F13 might arise entirely from precipitin reactions, while the grouping serum might have contained agglutinins which were interfered with by the mucoid capsules in the cases of antigens F1, F2 and F13, but which were permitted to exert their full action in the cases of G11 and G12, capsule-free organisms. The latter, then, would not be effective in removing by absorption the precipitins responsible for the F1-F2-F13 grouping. In such a case, an increase in the agglutination titer after hydrolysis of the capsule substance of F1, F2 and F13 would be expected. This has not been confirmed by us, although it is shown clearly in the results recorded by Beham.¹⁰

Precipitin Reactions.—The preparation of antigens for the precipitin reaction was attempted by two methods: by treating bacterial suspensions with HCl, heating to 80 C., afterward neutralizing with NaOH and centrifugalizing until clear; and by allowing cultures to grow in peptone solution at 37 C. for 30 days, and sedimenting by centrifugation. Difficulty was encountered in this procedure in that the cultures of the mucoid strains could not be centrifugated clear. After a first centrifugation, the opalescent supernatant fluid was drawn off in these instances, and a small amount of washed, ammonia-free aluminium hydroxide cream was added. After thorough mixing, such tubes yielded on subsequent centrifugation crystal clear extracts. An excess of the aluminum hydroxide is to be avoided, as it considerably lowers the amount of precipitinogen in the solutions. These methods yielded antigens equally effective. Serum and antigen were mixed, the results read after 1 hour at room temperature, and the readings confirmed after 18 hours in the icebox.

The results obtained with the precipitin tests are in accord with those of the agglutinin absorption tests, and may be presented briefly as follows:

Serum	Positive Results	
F2	F1, F2, and F13.....	All other strains negative
F6	F6	All other strains negative
F10	F10 and R1.....	All other strains negative
G1	None	All negative
G2	None	All negative
G3	None	All negative
G8	G8	All other strains negative
A1	A1	All other strains negative
R1	R1 and F10.....	All other strains negative

Strains G1, G2 and G3 yielded no precipitinogen in any of the extracts prepared by either of the two methods. These strains had become capsule-free on prolonged cultivation. The results of precipitin tests, while clear-cut, are less dependable than results by the absorption tests because of the failure of some strains to produce precipitinogen.

SUMMARY

In a study of the mucosus group of bacteria, 13 strains of respiratory origin and 11 from the lesions of inguinal granuloma were used. All were nonmotile, gram-negative, encapsulated bacilli exhibiting in cultures the characteristic colony growth of the mucosus group. Two stock strains of *B. aerogenes* and 1 of *B. rhinoscleromatis* were carried as controls.

The carbohydrate fermentation reactions were irregular and unreliable as a means of identification or of subgrouping the strains. Common immunologic strains showed diverse carbohydrate fermentation reactions.

None of the strains formed indol or creatinin from peptone, and none liquefied gelatin. All reduced nitrates and formed ammonia in a peptone medium. All produced amino acids from peptone, with the maximum concentration in the medium occurring after 6 to 9 days' incubation.

Granuloma strains were more resistant to the growth inhibiting effects of tartar emetic than were the respiratory strains, while the aerogenes strains occupied an intermediate position between these two.

A serologic study of 29 strains with immune serum prepared with 9 of them have revealed one group of 3 strains (F1, F2 and F13) and another of 2 strains (F10 and R1). Agglutination, absorption of agglutinins, and precipitin tests were used. Eight strains have been wholly unaffected by any of the 9 serums and 6 others have been agglutinated, apparently by group agglutinins, in low titers, each by a single serum. Of the 10 remaining strains, 7 have been shown to be individual immunologic strains, and 3 have been affected by group agglutinins by more than one serum. These 3 strains, with the 8 inagglutinable, and the 6 weakly agglutinable, strains comprise 17 strains which cannot be grouped by this study.

It is noteworthy that the grouping by serologic methods is entirely at a variance with that of the carbohydrate fermentation reactions, e. g., F1, F2 and F13 occur in one serologic group, while each occurs in a separate group by the carbohydrate fermentation reactions. Again, the stock rhinoscleroma strain appears identical immunologically with F10, a strain obtained from a pneumonic lung at necropsy, yet these strains differ greatly in their fermentation reactions.

CONCLUSIONS

Respiratory and granuloma strains of *B. mucosus* are not differentiated by the tests used, except possibly by the tartar emetic inhibition effects to which the respiratory strains appear more sensitive than do the granuloma strains.

Carbohydrate fermentation reactions should be discarded as a means of classification of bacteria of this group.

Lack of motility, presence of capsules, gram-negative staining, inability to produce indol from peptone, and the absence of gelatin liquefying properties are characters of this group which are most useful for purposes of identification. Inability to produce creatinin from peptone, ability to reduce nitrates, to form ammonia and amino acids from peptone are other characters common to the group. None of the characters are useful for purposes of identifying subgroups.

With the methods used in this study, serologic groups have been identified, but no practical classification of the *B. mucosus* group has been made.

TWO "FOOD POISONING" OUTBREAKS APPARENTLY DUE TO BACILLI OF THE PARATYPHOID ENTERITIDIS GROUP *

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(A) ROCKFORD, ILL.

On April 8, 1922, an explosive outbreak of gastro-enteritis affected 52 students of Rockford College, a college for girls at Rockford, Ill. The symptoms were similar to those usually recorded in such outbreaks: nausea, diarrhea and considerable prostration. Stools were of a soft to watery consistency, rarely bloody. Headache was common. Temperatures were not taken at the height of the outbreak, but in those patients in whom a moderate degree of prostration lasted for several days, subnormal temperatures were observed. Vomiting occurred in 10 out of 52 cases. There were no deaths.

We are under obligations to the Rockford College authorities, to Dr. Calhoun of the Rockford Hospital, and to Dr. Gunderson, the City Health Officer, for carefully verifying data and for cordial cooperation in our inquiry.

The time of onset and other features indicated that something eaten at the noon meal—12:30 to 1:30—on April 8 might have caused the illness. In several instances, symptoms appeared shortly before the evening meal; and 10 girls were so incapacitated that they did not come to the dinner table. In the others affected, symptoms appeared soon after eating dinner, that is, 7 to 8 hours after the suspected lunch. Several students who were ill in this outbreak do not eat at the College except at noon.

The food served at what was apparently the causative meal consisted of a veal loaf with cream sauce, commercially canned peas, mashed potatoes, baking powder biscuits, coffee and milk. The veal loaf with the cream sauce was the only article of food served to all those who were ill. Several persons who ate this dish, however, were not affected.

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Since veal loaf and similar dishes have been rather frequently implicated in food poisoning cases, careful inquiry was made about details of preparation. It was found that the veal loaf was made from government inspected beef, veal and pork received at the college March 31, April 3 and April 5, respectively, and placed at once in the excellent refrigerating plant. The beef was cooked and served as roast on April 4, the veal on April 6 and the pork on April 7. The remaining portions were ground up together and made into the veal loaf served on April 8. The loaf was cooked for one hour. A portion of the loaf which remained was examined on April 8, and appeared well cooked throughout, normal in appearance, odor, taste, and texture. A bacterial examination showed nothing significant.

The cream sauce was prepared by placing the milk in a "steam table" for about 3 hours, thickening with flour and butter and then keeping warm for about 2 hours longer. The actual temperatures used in this process were indefinite, but were unquestionably below the thermal death point of the organisms of the paratyphoid group. The point was tested experimentally. Sauce prepared in the same manner, after inoculating the milk with *B. coli*, showed that *B. coli* survived throughout the process of manufacture.

That the cream sauce rather than the veal loaf was the cause of the trouble was indicated by several considerations. Two other outbreaks of food poisoning at Rockford College had been suspected by the college authorities of a connection with cream sauce. The first of these occurred in March, 1921, when cream sauce had been used on asparagus, and the second in October, 1921, when a similar sauce had been used on custard. The causative meal in both these instances was dinner, and in correspondence with the larger number of persons served (about 200), the number affected (March, 119, October, 121) was greater than at the luncheon in April (52). According to the records of the College, 43 persons were ill in all 3 outbreaks.

Cultures of 2 organisms isolated the day after the April, 1922, outbreak, 1 from the stools of a patient, 1 from the milk supply, were regarded as possibly significant. These cultures were identical, and when examined by us proved to be typical *B. paratyphosus* A, attacking arabinose quickly, dulcitol slowly and xylose not at all. Serologically, as well as culturally, they agreed completely with the members of a carefully tested group of these organisms.¹

¹ Jordan: Jour. Infect. Dis., 1917, 20, p. 451.

The milk that served as a basis for the cream sauce was obtained directly from a pasteurizing plant, but no automatic records of the temperatures maintained were available.

If the contaminating organisms were present in the milk, as it came from the pasteurizer, other cases of "food poisoning" might have been expected to occur among the users of milk from this source. In point of fact, similar cases are known to have occurred at about the same time as the last college outbreak among the patrons of a public restaurant in which a cream sauce was served, made with milk from the incriminated dairy.

Since *B. paratyphosus* A is not usually found in connection with infections of the lower animals, a careful search for human carriers was made. The facts already cited made it appear improbable that contamination of the milk or sauce had occurred within the college itself, and this opinion found confirmation in the circumstance that the kitchen personnel in the college was different at the time of each of the 3 outbreaks. A single bacterial examination of the stools of the kitchen employees after the April outbreak was made by us, but was negative. The results of a survey of the sources of milk supply were no more successful. Inquiry at the homes of milk producers elicited no history of disease that could be regarded in any way significant. A single bacterial examination of the stools of the employees of the pasteurizing plant gave nothing positive.

Before the occurrence of the first of these 3 outbreaks, 25 students had been inoculated against typhoid, 15 with the "triple vaccine" (*B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B); 10 of the latter had been inoculated within 2 years. Among those inoculated, only 3 escaped all 3 outbreaks. The serum of one girl who was ill in both the second and third outbreaks was found after the April outbreak to agglutinate *B. typhosus*, *B. paratyphosus* A, and *B. paratyphosus* B.

The serums of 22 students who were ill in the April outbreak were obtained 6 weeks later and were tested for agglutinins. One of these students had been inoculated with the "triple" typhoid vaccine, and the results are consequently of little comparative value. Table 1 gives the agglutination reactions for thirteen students.

Serums from 8 other uninoculated students gave entirely negative agglutination results in 1:40 dilution with the organisms specified in the table; 1 of these students had been ill in the first outbreak, 1 in the second, 3 in both the first and the second, and 5 in all 3 outbreaks.

It seems, in conclusion, a plausible assumption that in this outbreak of food poisoning the milk supply serving as the basis in the preparation of a cream sauce became contaminated with *B. paratyphosus* A from a human carrier (exact source unknown), and that the poison elaborated under the incubating conditions maintained in the preparation of the sauce was the direct cause of the symptoms. Intoxication rather than infection seems probable because of the relatively transient character of the symptoms and the lack of definite agglutinin formation in the blood of those affected.

The features of special interest in this outbreak of food poisoning are: (1) the occurrence of 3 outbreaks, apparently due to a "cream sauce," in substantially the same group of persons; (2) the isolation

TABLE 1
THE AGGLUTINATION REACTIONS OF THE BLOOD SERUMS OF THIRTEEN UNINOCULATED PERSONS WHO WERE ILL IN THE ROCKFORD OUTBREAK

Names	B. Typhosus			B. Para-typhosus A Milk			B. Para-typhosus A Stools			B. Para-typhosus B		
	1:40	1:80	1:160	1:40	1:80	1:160	1:40	1:80	1:160	1:40	1:80	1:160
M.	+	+	+	..	+
X.	+	+	+	+
Z.	+	+	+	+	+	+
P.	+	+	+	+	+	+
R.	+	+	+
B.	+	+
A.	+	+	..	+	+	..
W.	+	+
L.	+	+	..	+	+
W.	+	+	..	+	+	..	+
M.	+	+	..	+	+	+
B.	+	+	+	+	..
J.	+	+	+	+	+	+	..

of *B. paratyphosus* A from the milk used in making the sauce and also from the stool of 1 of the persons affected; (3) the apparent lack of an immunization process, as evidenced by the repeated attacks experienced by one and the same person.

(B) BIRMINGHAM, ALA.

On the afternoon of Sept. 27, 1922, several hundred pupils of the Woodlawn High School, Birmingham, Ala., exhibited suddenly and almost simultaneously symptoms of violent gastro-intestinal disturbance. Projectile vomiting was one of the most conspicuous symptoms; diarrhea was present in nearly one-third (112) of the total number affected (393). Abdominal pain was experienced by many before the objective symptoms were manifested. A number of the pupils expressed their

belief that a slight degree of fever accompanied the attack, and in the few patients attended by physicians a slight elevation of temperature was recorded. The majority of those affected recovered within 48 hours, and the roll call of the high school 5 days after the outbreak showed the attendance to be practically normal. In no case was any continued pain or illness observed, and there were no fatalities.

We are under obligations to Dr. J. D. Dowling, Health Commissioner, and to Dr. J. S. McLester, Director of Health, of the Birmingham public schools, for the valuable assistance they gave us in the course of our inquiries.

The limitation of the outbreak to the pupils of the Woodlawn school focused attention on the school lunch as a possible factor. Pain in the abdomen was noticed by many within about 1½ to 2 hours after the school lunch, but manifestation of the characteristic symptoms reached its height between 3 to 4 hours after the suspected meal.

The lunch at which the presumably causative food was served consisted of a meat sandwich and a glass of raw milk, for which a nominal charge was made. Other items for which an additional charge was made were peanut butter sandwiches, soup, bread and butter, buns, chocolate cake, iced tea and lemonade. The only foods partaken of by practically all those who were ill were the meat sandwich and the milk.

The milk, like most of the Birmingham supply, was unpasteurized, but the average bacterial count was not high, 3 samples that were examined averaging 6,000, 4,000 and 24,000 colonies per cc., respectively. The school received 10 gallons of this milk daily, 8 gallons in cans, the remainder in bottles. The same dairy supplied about 130 gallons of milk daily to the residents of the district in which the school is located. No illness is known to have occurred at this time among these outside users of the milk.

The history of the meat sandwich pointed to the latter as a more likely source of trouble. The meat was government inspected beef and was purchased from a reputable market. It consisted of parts of 2 hind quarters cut into 3 sections, and weighed about 60 pounds. It arrived at the market from the government inspected plant on September 25, was placed in the ice chest, and was sent to the school in a covered basket on the morning of September 26. It was then washed in tap water and was boiled for a time, estimated to be from 2½ to 3 hours. The liquor from this meat was used for soup the next day. As already stated, no illness could be attributed to the use

of the soup. The meat, after boiling, was cut into small sections by the kitchen personnel at the Woodlawn school, presumably ground up while yet warm, placed by hand in large earthenware bowls and put into the stove until the following day, the day of the outbreak, September 27. From what could be learned, it seemed probable that the stove was warm at the time the meat was placed inside. At this time the atmospheric temperature in Birmingham was high, reaching a maximum of 88 F. on the 26th and 85 on the 27th. From these facts there seems little doubt that the ground meat after handling was kept at a highly favorable incubating temperature for a considerable period. Of the 393 pupils who were ill, 383 are known to have eaten the meat sandwich. It was thought that possibly the 10 pupils who showed signs of illness, but gave no history of having eaten the meat sandwich might have been sympathetically affected by the devastating scenes with which they were surrounded. Of those not ill, but who ate certain articles of food at the lunch, exact numerical data could not be obtained. There is no doubt that some persons (25 to 35?) who ate the meat sandwich were not ill. A much larger number (100?) who did not eat the meat sandwich remained unaffected.

While the results of the epidemiologic inquiry were not entirely conclusive, additional evidence obtained by bacterial examination appeared to fix responsibility for the outbreak more definitely on the meat sandwich. Only one sandwich was recovered for examination. This was obtained from a teacher by Dr. Dowling, City Health Officer, and was turned over to us in Birmingham on September 30. It had then been in the ice chest for 3 days. The meat, which was finely ground and uniformly spread between 2 thin slices of white bread, amounted to several grams in weight. Both meat and bread were very dry. A portion of the meat was suspended in sterile water and plated directly in Endo medium; the remainder was forwarded to Chicago for further examination. The colony count, as might be expected, was very high, amounting to about 70,000,000 per gram. Colonies resembling those of the paratyphoid group were picked from the Endo medium plates in Birmingham and transferred to tubes of Russell medium, which were taken to Chicago. From the tubes which were brought to Chicago several cultures of a paratyphoid bacillus were isolated. These proved to be identical and to resemble *B. paratyphosus* B (*B. schottmülleri*), both culturally and serologically.² When

² *Ibid.*, p. 457.

first isolated, this strain did not agglutinate to the full titer with the type B. paratyphosus B serum, but after a few test tube generations it showed complete identity with the other members of the laboratory collection when tested directly and also by the absorption method. Paratyphoid bacilli could not be isolated from the portion of the meat sent to Chicago.

Specimens of stools from 26 different persons who were affected in the outbreak were examined. These were obtained 7 days after the outbreak. Although this material was subjected to searching examination, no paratyphoid organism was isolated in any instance.

A considerable number of the pupils of the Woodlawn school had been inoculated with the "triple" typhoid vaccine prior to the outbreak of September 27. No effect on the incidence of the illness could be attributed to this inoculation, as shown by the following figures:

	Ill	Not Ill
Inoculated	178	67
Not Inoculated	193	77

Twenty serums from uninoculated persons who were affected in the outbreak were tested for agglutinative power on 4 cultures: B. typhosus, B. paratyphosus A, a laboratory strain of B. paratyphosus B and the strain of B. paratyphosus B isolated from the meat sandwich. None of the serums in 1:80 dilution had any effect on the 3 strains from the University of Chicago laboratory collection. Four serums in 1:80 dilution had no effect on the Birmingham organisms and 3 others in the same dilution showed only a trace of agglutinative action on this strain. The remaining 13 serums in 1:80 dilution had a definite positive reaction with the Birmingham sandwich strain; 4 of these showed agglutination with a dilution of 1:160 and 2 as high as 1:320. Fresh specimens of serums from 4 of the persons showing the highest agglutination were obtained 3 months later (Jan. 3, 1923), and tested against all 4 cultures: none of them produced any agglutination in 1:80 dilution with any culture, including the Birmingham strain.

It seemed a plausible supposition that the meat, which had been handled by a number of the kitchen personnel during the process of preparation, might have been contaminated by a paratyphoid carrier and that the particularly favorable condition of incubation had led to an enormous multiplication of the specific organisms found in the sandwich with consequent toxin production. No history of illness

among the kitchen employees, however, could be elicited. Stool samples from these employees were obtained 7 days after the outbreak, but the bacterial examination was negative.

Although there are noticeable gaps in the evidence, it seems probable that the Birmingham food poisoning outbreak was due to the specific paratyphoid organism (*B. paratyphosus* B) found in the meat, since organisms of this type have been frequently implicated in similar outbreaks, and since the epidemiologic data seemed to point to the meat as the peccant food. The exact source of contamination of the meat could not be discovered.

It may be noted that in both these outbreaks previous inoculation with a vaccine containing the typhoid bacillus and strains of paratyphoid bacilli (A and B) failed to confer any protection. It is evident also that if these outbreaks were due, as seems probable, to poisonous substances produced by bacilli of the paratyphoid group, diagnosis of the causal agent in such cases cannot safely be made from agglutination tests with the patient's serum. In many instances, no agglutinins were found in the serum of those affected; and in others, the agglutinative reaction was as strong with other bacilli of the group as with those apparently causally related. In spite of the lack of absolute uniformity and certainty, however, it is worth noting that in both outbreaks the specific bacilli isolated were agglutinated in higher titer and by a larger proportion of serums than the other organisms tested.

FINAL HYDROGEN-ION CONCENTRATION IN THE PARATYPHOID ENTERITIDIS GROUP

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The degree of hydrogen-ion concentration in bacterial culture mediums at which growth stops differs somewhat in different groups of micro-organisms. In the colon-aerogenes group, differences in final hydrogen-ion concentration are correlated with other characters distinguishing the *B. coli* and *B. aerogenes* types. Hemolytic streptococci from human sources are limited by a lower hydrogen-ion concentration than those from bovine sources.¹ Pneumococci, however, in this respect do not show any noteworthy divergence from human streptococci.²

Access to Professor Jordan's collection of carefully verified strains³ has given me an opportunity to determine the final hydrogen-ion concentration in the paratyphoid-enteritidis group with a view to discovering the possible differential value of the reaction.

The technic has been used by most of the later workers in this field, e. g., Foster.⁴ Care has been taken in the preparation of glassware, reagents, etc., to avoid possible sources of error. Beef infusion 1% dextrose peptone broth with an initial P_H of 8.0 has been used as the culture medium. An initial P_H of 7.3 was used for comparison of some strains without any essential difference in the results. The cultures (80 cc.) have been held at 37 C. in pyrex flasks. The readings for final hydrogen-ion concentration have been taken after 7 days, but there is rarely any change after 48 hours. In all comparative tests, the buffer content of the mediums has been uniform. Since the results of these experiments have been negative so far as any differentiation

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¹ Avery and Cullen: *Jour. Exp. Med.*, 1919, 29, p. 215; J. H. Brown: *Ibid.*, 1920, 30, p. 35.

² Avery and Cullen: *Ibid.*, 1919, 30, u. 357; Lord and Nye: *Ibid.*, p. 387; H. M. Jones, *Jour. Infect. Dis.*, 1920, 26, p. 435.

³ *Ibid.*, 1917, 20, p. 457.

⁴ *J. Bacteriol.*, 1921, 6, p. 161.

within the paratyphoid-enteritidis group could be noted, they are summarized briefly:

Types	Number of Strains Tested	Range of P_H
<i>B. paratyphosus</i> A.....	13	4.8
<i>B. paratyphosus</i> B.....	25	4.6-4.9
<i>B. paratyphosus</i> C.....	4	4.7
<i>B. enteritidis</i>	7	4.6-4.9
<i>B. suispestifer</i>	18	4.6-4.9

At the end of the 7-day period, all cultures were tested for viability. In no instance was growth obtained.

For comparison, 12 strains of *B. typhosus*, 5 of dysentery bacilli (1 "Shiga," 1 "Y," 1 "Strong" and 2 "Flexner") 5 of *B. coli* and 1 of *B. (lactis) aerogenes* were grown in a similar manner. *B. aerogenes*, although reaching a P_H of 5.1 after 24 hours, showed an increase to P_H 7.7 at the end of 7 days in the manner made familiar by previous investigators. All the others, with the exception of 3 strains of the mannitol-fermenting dysentery type, had a final hydrogen-ion concentration close to 4.8. One of the 4 mannitol-fermenting strains ("Flexner") behaved like the typhoid and paratyphoid bacilli in this particular, but the other 3 gave consistently higher P_H values (5.2-5.6). One of these strains ("Strong") was always viable at the end of 7 days and 1 of the other (Flexner H) sometimes, in this respect contrasting with all the typhoid and paratyphoid strains. The *B. aerogenes* cultures were always viable after 7 days and 1 or more of the 5 *B. coli* cultures also usually survived.

SUMMARY

The several types of paratyphoid-enteritidis bacilli cannot be differentiated from one another or from the typhoid bacilli by the final hydrogen-ion concentration in dextrose broth cultures. The P_H varies within rather narrow limits (4.6-4.9). Rarely is a viable culture found at the end of 7 days.

Certain strains of dysentery bacilli have on the average a somewhat higher P_H value after 7 days (5.2-5.6), and in this respect as well as in persistent viability, stand closer to *B. aerogenes* than do the other members of the group.

EXPERIMENTAL GANGRENE

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The majority of attempts to produce gangrene in animals with spirochetes and fusiform bacilli have failed. Tunnicliff,¹ who studied these organisms carefully, failed to produce gangrene in dogs, rabbits, guinea-pigs, white rats and pigeons following the injection of cultures of spirochetes and fusiform bacilli subcutaneously, intraperitoneally, intravenously and into the mucous membrane of the mouth. On the other hand, Veszpremi² in 1907 reported the successful production of gangrene in rabbits following subcutaneous inoculation, the material containing spirochetes and fusiform bacilli and a third organism known as *Cladothrix putridogenes*. A considerable number of his animals, however, showed only abscess formation. In one of the latter animals an abscess was incised, and in a short time the lesion became gangrenous, leading Veszpremi to the belief that the actual gangrenous character of the lesion depended on aerobic rather than anaerobic organisms. Veszpremi stated it as his belief that when the viability of tissue is diminished by disease, the organisms living in the neighborhood, including *Spirochaeta gracillus*, increase enormously in number, there is an increase in the virulence of the organisms also and the process takes on a progressive character with the penetration of spirochetes into the tissue prepared for it. The spirochetes are aided by the fusiform bacilli and under the influence of the cladothrix and other bacteria, the characteristic foul-smelling gangrene follows.

METHODS EMPLOYED IN PRODUCING GANGRENE

Since it is commonly accepted that gangrenous necrosis occurs only in tissue previously devitalized, it was decided to traumatize tissue before inoculating the material containing spirochetes and fusiform bacilli. Accordingly, a guinea-pig was anesthetized, the hair plucked from the proximal portion of each hind extremity and the tissues on one side clamped repeatedly with a hemostat. The other side was left untraumatized. This first animal was inoculated intramuscularly on each side

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¹ Jour. Infect. Dis., 1911, 8, p. 316.

² Centralbl. f. Bakteriol., I, O., 1907, 44, pp. 332, 408, 515, 648; 1908, 45, p. 15.

with 1 cc. of fluid from a gangrenous lung containing innumerable spirochetes fusiform bacilli, and other bacteria. The animal died within 2 days and showed extensive greenish brown gangrenous necrosis on the traumatized side; the odor was foul, penetrating. There was also a small area of gangrenous necrosis on the untraumatized side. The gangrene in this case was apparently identical in character with that observed in the patient from which the organisms were obtained. This procedure was followed in a number of guinea-pigs with resultant typical gangrenous necrosis on the traumatized side in 78% of them. In the failures the majority showed abscess, pus from some of which in other traumatized guinea-pigs produced typical gangrenous necrosis. Three rabbits were injected intrabronchially through a treacheotomy wound with spirochetes, fusiform bacilli and other organisms. In 2, gangrenous pulmonary lesions resulted, in 1 a pneumonia with areas of necrosis not gangrenous resulted. The guinea-pigs died, usually within 48 hours; 2 rabbits died in 9 days, the 3rd rabbit was killed on the 9th day. It is believed that the failures resulted from technical errors, principally insufficient trauma of tissue, that too few organisms were injected, or that the material was exposed to the air too long before inoculation. The material producing gangrene was derived from 2 cases of dental caries and pyorrhea alveolaris, 3 cases of pulmonary gangrene, 4 cases of Vincent's angina and over 40 cases of experimentally produced gangrene in guinea-pigs. The gangrenous necrosis produced in the animals was apparently the same regardless of the source of the spirochetes and fusiform bacilli.

The experiments can be conveniently divided into 3 groups comprising 62 animals. In one group, material from pulmonary gangrene containing innumerable spirochetes, fusiform bacilli and other organisms was passed from guinea-pig to guinea-pig producing typical gangrenous necrosis in the 36th generation. The spirochetes diminished rapidly in the guinea-pigs, disappearing entirely after passage through 12 animals. The fusiform bacilli, however, were present in all cases of gangrene. In another group of experiments material from a gangrenous pulmonary lesion containing innumerable spirochetes, fusiform bacilli and other organisms was injected into 2 guinea-pigs in the routine manner and into 1 rabbit intrabronchially. From the same case material from a carious tooth showing organisms apparently similar to those in the gangrenous lung was injected also into 2 guinea-pigs in the usual manner and into 1 rabbit intrabronchially. The guinea-pigs all died in from 2 to 4 days, and all 4 showed marked typical gangrenous necrosis on the traumatized

side. The 2 rabbits died in 9 days, and both showed extensive gangrenous pleuropulmonary lesions. In the 3rd group of experiments, material from 4 cases of Vincent's angina containing spirochetes and fusiform bacilli was injected in the routine manner into 4 guinea-pigs, resulting in typical gangrene in 3; in the 4th, a putrid abscess resulted. The material from this abscess, however, injected into a traumatized guinea-pig, resulted in typical gangrenous necrosis a few cm. in diameter. That gangrene may represent merely a more severe lesion than these organisms produce, is further evidenced by the following experiment: Two guinea-pigs were injected with material from experimental gangrene containing fusiform bacilli but no demonstrable spirochetes. In each case, an abscess developed in the inoculated area. On the seventh day of the experiment in one case and on the eleventh in the other, the affected extremity was traumatized in the routine manner. One animal died 2 days after the trauma, the other 3 days after. In both cases, there was marked typical gangrenous necrosis in the involved area, smears from which showed not only a great many more fusiform bacilli than in the injected material, but also spirochetes of the Vincent type as well, in one case in large numbers, in the other in relatively small numbers.

CONCLUSIONS

In the presence of devitalized tissue, gangrene may be produced in guinea-pigs with material from dental caries and pyorrhea alveolaris, Vincent's angina and pulmonary gangrene containing spirochetes and fusiform bacilli.

(2) The experimental production in the rabbit of pulmonary gangrene with material from a carious tooth containing spirochetes and fusiform bacilli is further evidence that the aspiration of these organisms from lesions in the mouth of human beings may lead to pulmonary gangrene.

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